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PLATELET FACTOR 1 RELATED TO PROTHROMBIN ACTIVATION[1]

COLIN FELL[2] AND WALTER H. SEEGERS[3]

Abstract

Concentrates of platelet factor 1 were made from bovine platelets. Being bound to fine particles, the activity was not obtained in solution. The activity of the concentrates is destroyed by heating at 53° C., at pH 5.2, by extraction with ether, by drying with acetone, or by standing at −20° C. The concentrates accelerated the clotting of whole blood, and also the activation of prothrombin either to form thrombin or a non-thrombin derivative. Activation of purified prothrombin occurred under several different circumstances, and different end products formed. Platelet factor 1 concentrates appear to have a general property of accelerating alterations in the prothrombin molecule independently of these variants.

Introduction

Platelets contain material which accelerates the conversion of prothrombin to thrombin. The active material is thermolabile, non-dialyzable, and sedimentable at 32,000 *g*, and has been referred to as platelet factor 1 or platelet Ac-globulin (1–3). Subsequent investigators (4–6) have confirmed that platelets have this activity. Of particular interest was the work of Deutsch (7), and Deutsch, Johnson, and Seegers (8), where platelet factors 1, 2, 3, and 4 were separated from one another. Advantage was taken of differences in stability, solubility, and particularly of sedimentation characteristics upon centrifugation. Platelet factors 1 and 3 were readily sedimented in an ultracentrifuge, and platelet factor 1 was found to be more stable, in the preparations used, than platelet factor 3. The platelets of a parahemophilic patient (congenital deficiency of plasma Ac-globulin) did not contain the accelerator in appreciable quantity (9–11), but these platelets acquired it if suspended in plasma from a normal person (11).

In the present work we prepared concentrates that contain platelet factor 1 activity, investigated some of their properties, and related the activity to

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Contribution from the Department of Physiology and Pharmacology, Wayne State University College of Medicine, Detroit, Michigan. This investigation was supported by a research grant H-2026 from the National Heart Institute, National Institutes of Health, U.S. Public Health Service. Funds for Research Fellowships in Physiology were provided by Parke, Davis and Company.

[2]Present address: Department of Physiology, Medical College of Georgia, Augusta, Georgia.

[3]Work completed during tenure of Award in Support of Creative Work from the Commonwealth Fund of New York.

prothrombin activation. We did not obtain the activity in solution; it was firmly bound to platelet particles. The concentrates accelerated the clotting of whole blood. Moreover, under several conditions where purified prothrombin activates, the concentrates accelerated the activation. This included transformation to prothrombin derivative as well as to thrombin.

Materials and Methods

The preparation of imidazole buffer (12), bovine platelet concentrates (13), platelet factor 3 (14), $BaCO_3$ eluate (material adsorbed from bovine serum and eluted) (13), brain extract thromboplastin obtained as saline extracts of acetone-dried rabbit brain (15), lung extract thromboplastin (bovine) (6), lung thromboplastin (bovine) (16), and citrate thrombin (17) have been previously described. Assay procedures for thrombin (18), prothrombin (19), Ac-globulin (20, 21), and platelet factor 3 (14) have also been described. The method of Folin and Ciocalteau (22) was used for "tyrosine" determinations (the procedure is not entirely specific for tyrosine).

All experiments were performed repeatedly. Finally the data for the text figures were obtained with all reagents especially checked for standard quality. No statistics were used. Each point on a curve is usually an average of three or four analyses. The curves were drawn by inspection.

Purified prothrombin was prepared from bovine plasma (23). In most cases it was heated 2 hours at 53° C. to remove traces of Ac-globulin (24). Platelet cofactor I was made from bovine plasma as described previously (25). The bovine fibrinogen used in all assay procedures was purchased from Armour and Company. Brain thromboplastin (bovine), free of protein, was prepared according to Hecht, Cho, and Seegers (26).

A convenient one-stage assay for platelet factor 1 was developed on the basis of using stored oxalated human plasma as a substrate. The following reagents were mixed: 0.1 ml. of 0.032 *M* $CaCl_2$; 0.1 ml. stored oxalated human plasma (low in Ac-globulin); 0.1 ml. brain extract thromboplastin (rabbit); and 0.1 ml. physiological saline solution. The latter served as solvent for the unknown. The concentration of platelet factor 1 was expressed in units. As the concentration of Ac-globulin or the platelet accelerator increased, the clotting times shortened. $BaCO_3$-adsorbed bovine serum (19) served as reference standard and was assigned the value of 100 units/ml. This was a convenience even though analysis by the more complicated two-stage procedure of Ware and Seegers (21) indicated a concentration of 66 in terms of their units.

Experimental

Concentration of Platelet Factor 1

The various manipulations were designed to disrupt the platelets and to disperse the fragments in fine suspension. To concentrate the platelet factor 1, advantage was taken of its ready sedimentability in an ultracentrifuge. Platelets were broken up by freezing a suspension of one part of packed platelets

in four parts of physiological saline solution, thawing the mixture, and homogenizing with a hand homogenizer. The suspension was then centrifuged 20 minutes at 2000 *g*, and the sediment was again extracted with the original volume of imidazole buffered saline. The first extract was then simply added to the second, the latter still containing particulate matter. Citrate thrombin was added to a final concentration of 2 units/ml. to remove the clottable factor present in platelet extracts (2). There was no loss of activity associated with its removal. Actually some increase was usually noted. The coagulum was broken up with a stirring rod and removed along with the grosser platelet fragments by concentrifugation at 2000 *g* for 20 minutes. The sediment was washed one or more times with twice its volume of buffered saline, and centrifuged. The supernatant "solutions" were added to the first extracts. All combined extracts were then centrifuged 1 hour at a minimum of 35,000 *g* to obtain the active material as a sediment. The latter was washed once with buffered saline and centrifuged again. The washed sediment was suspended in buffered saline. The volume usually represented a fifth that of the starting material. The final products were milky white, and opalescent. The particles did not settle on prolonged standing in glass test tubes at 25° C.

Properties of Platelet Factor 1 Concentrates

After preliminary work we made 19 preparations and found considerable variation from one product to another. In general, the concentrates were two to six times as active as the packed platelets in four parts of solvent used as starting material. The average concentration was about 4 units/ml. Specific activities ranged between 9 and 44 units/mg. of "tyrosine", representing an increase in purity ranging from 5- to 40-fold. Calculations of yield indicated a recovery of 50 to more than 100%. High yields presumably reflect release of material or greater dispersion of the active particles.

The concentrates in no sense represent purified material. The products were free of thrombin, and there was no platelet factor 2 activity. Platelet factor 4 (antiheparin activity) was present in much lower concentration than in the original platelet suspensions. There was a variable but consistently high amount of platelet factor 3; for example, one product had 1575 units/ml. By contrast purified platelet factor 3 preparations made as described (14), do not contain platelet factor 1.

The stability of concentrates at 4° C. was quite variable, but in general the activity kept well over a 2 week period, disappearing gradually thereafter. At 28° C. the activity disappeared within 48 hours, and attempts to retard presumed bacterial growth with thymol or toluene hastened this disappearance. Platelet factor 1 of our preparation was not sedimented by centrifugation at 2500 *g* for 30 minutes. It was partially sedimented at 10,000 *g*, and was almost completely sedimented at 26,000 *g*. It did not pass through a cellophane dialysis membrane. The activity was destroyed completely by heating at 53° C. for 2 hours, and on standing for 1 hour at pH 5.2. It disappeared entirely on standing 24 hours at −20° C. On the other hand snap freezing with a mixture of dry ice and alcohol and rapid thawing for 10 successive times

destroyed about 65% of the activity. Extraction with ether, and drying with acetone destroyed all the activity. Mixing with petroleum ether or lyophilization or precipitation with 30% (v/v) cold ethyl alcohol were less destructive, but considerable activity was lost. Fractionation with ammonium sulphate at 0° C. and at 60% of saturation was unsuccessful. Adsorption with $BaCO_3$, kaolin, and $Ca_3(PO_4)_2$ removed platelet factor 1 completely, leaving water-clear supernatant solutions. Attempts to elute the activity were unsuccessful.

Activation of Purified Prothrombin with Platelet Factor 1 Concentrate

As in the work of Ware and Seegers (24) we found that prothrombin (free of Ac-globulin) was activated when mixed with lung thromboplastin or brain thromboplastin and calcium. About 30% of the prothrombin became thrombin (Fig. 1) and a greater portion changed to a prothrombin derivative that did not become thrombin in the two-stage analytical reagents. Some prothrombin remained. Addition of platelet factor 1 concentrate considerably increased the amount of thrombin obtained, and the rate of thrombin formation (Fig. 1). The remaining prothrombin became a non-thrombin derivative. Since our preparation was not adequate to create the conditions for changing all the prothrombin substrate to thrombin, we added an eluate of material adsorbed from bovine serum ($BaCO_3$-eluate). Under suitable conditions these eluates are potent adjuncts in the conversion of prothrombin to thrombin (13). With the platelet factor 1 and the eluate combined the yield was 90% of the value obtained by a two-stage analysis of the prothrombin substrate.

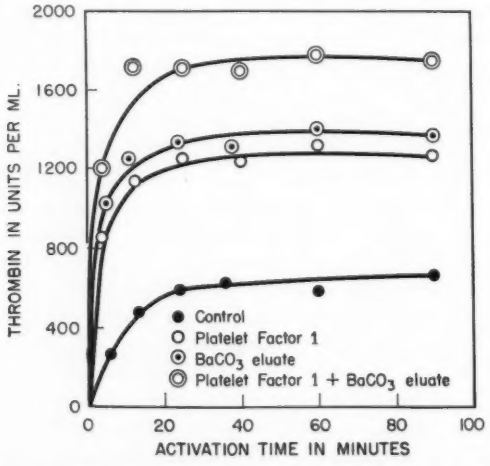

FIG. 1. Activation of purified prothrombin (2000 units/ml.) free of Ac-globulin in solutions with calcium ions (0.005 *M*), lung thromboplastin (4 mg./ml.), $BaCO_3$ eluate, and platelet factor 1. Some thrombin forms in the presence of calcium and thromboplastin (control). Addition of platelet factor 1 (0.7 unit/ml.) or $BaCO_3$ eluate (1.7 mg./ml.) increases the rate of thrombin formation, and the yield. Platelet factor 1 and $BaCO_3$ eluate combined are more potent in this regard than either alone.

Conversion of Prothrombin to Prothrombin Derivative

In mixtures of lysed platelets, calcium ions, and purified prothrombin, the latter transformed to a derivative (27). This derivative did not become thrombin upon analysis with the two-stage prothrombin analytical technic. The material in platelets which enables this kind of prothrombin activation to occur is platelet factor 3 (28). We turned attention to what was not previously a subject of inquiry; namely, that the loss of prothrombin activity occurred even when the prothrombin and platelet factor 3 did not contain Ac-globulin (Fig. 2). Moreover, the change prothrombin → prothrombin derivative was associated with the production of very little thrombin. Platelet factor 1 accelerated the change of prothrombin to derivative (compare curves of Fig. 2).

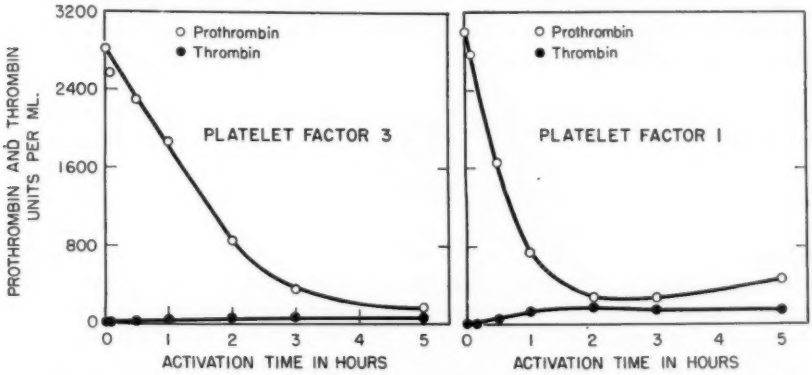

FIG. 2. Activation of purified prothrombin free of Ac-globulin in solutions with calcium ions (0.005 *M*) and platelet factors. With platelet factor 3 (100 units/ml.) very little thrombin forms; prothrombin alters so that it is neither prothrombin nor thrombin. Platelet factor 1 (0.2 unit/ml., and providing 100 units/ml. of platelet factor 3) accelerates derivative formation.

Conversion of Prothrombin to Thrombin

In the previous experiment we studied the activation of prothrombin according to the following equation:

$$\text{prothrombin} \xrightarrow[\text{platelet factor 3}]{Ca^{++}} \text{prothrombin derivative.}$$

When platelet factor 1 was added the rate of the reaction was increased. In other work from this laboratory (29) it was found that the above equation can be modified by adding thrombin at zero time to give:

$$\text{prothrombin} \xrightarrow[\text{platelet factor 3}\\ \text{thrombin}]{Ca^{++}} \text{thrombin.}$$

Moreover, another possibility for transforming the prothrombin to thrombin (29) is according to the following equation:

$$\text{prothrombin} \xrightarrow[\text{thrombin}]{\text{Ca}^{++},\ \text{platelet cofactor I},} \text{thrombin.}$$

According to the last equation, however, the yield of thrombin was not easily brought to a higher value than 50% even if the platelet cofactor I concentration and thrombin concentration were high (29). (This whole discussion is related to a time interval of less than 2 hours. If the time of observation is extended the circumstances are again different.) With these facts in mind we used relatively small amounts of thrombin, platelet factor 3, and platelet cofactor I to determine whether platelet factor 1 would make a difference according to the following equation:

$$\text{prothrombin} \xrightarrow[\text{platelet factor 3, platelet factor 1, thrombin}]{\text{Ca}^{++},\ \text{platelet cofactor I,}} \text{thrombin.}$$

The concentration of factors in units per milliliter was as follows: prothrombin, 2000; platelet cofactor I, 17; platelet factor 3, 100; and thrombin, 17. This

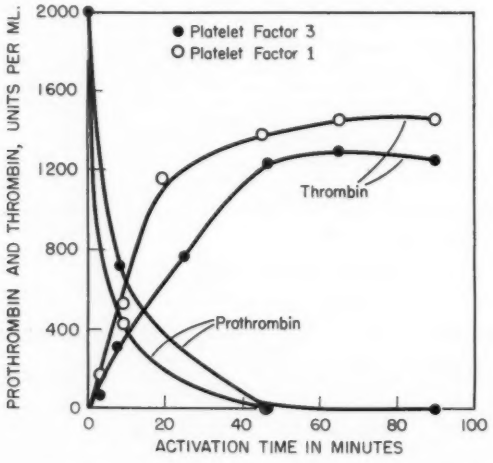

FIG. 3. Activation of purified prothrombin free of Ac-globulin in solutions with calcium ions (0.005 *M*), platelet cofactor I (17 units/ml.), thrombin (17 units/ml.), and platelet factors. With platelet factor 3 (100 units/ml.) prothrombin activity disappears, and considerable thrombin is formed. With platelet factor 1 (0.2 unit/ml., and providing 100 units/ml. of platelet factor 3) thrombin generation is accelerated.

mixture gave a thrombin yield of 65%. Then platelet factor 1 (accelerator) was added to give a concentration of 0.2 units/ml. Thrombin activity evolved more rapidly and the yield was 74%.

Clotting of Human Blood

Whole blood (0.8 ml.) added to physiological saline (0.2 ml.) clotted in glass in 600 seconds and in silicone tubes in 1620 seconds. With purified platelet factor 3, supplied in 0.2 ml. and to give a final concentration of 105 units/ml., the clotting times were respectively 410 and 705 seconds. With 0.3 unit platelet factor 1 and 90 units platelet factor 3 the clotting times were 120 and 145 seconds. The platelet factor 1 is thus an accelerator in whole blood as well as in the activation of purified prothrombin.

Discussion

Platelets contain a hypothetical substance called platelet factor 1 or platelet accelerator. It is recognized in terms of its ability to accelerate the clotting of blood, or by shortening the prothrombin time of stored plasma. Our attempts to free this activity from easily sedimentable material have not been successful. Concentrates have been obtained, but not of sufficient quality to obtain information about characteristics on a molecular basis. Consequently, we do not know whether this is a single substance.

The accelerator properties of platelet factor 1 concentrates are evident in the activation of purified prothrombin (free of Ac-globulin). When the activation of prothrombin involves formation of thrombin, platelet factor 1 concentrates accelerate the conversion. This is true whether lung thromboplastin or brain thromboplastin is used, or whether platelet factor 3 plus platelet cofactor I are used. When the activation involves formation of a non-thrombin derivative, platelet factor 1 concentrates again accelerate the conversion. The general changes facilitated are those which can occur in prothrombin itself.

References

1. Mann, F. D., Hurn, M., and Magath, T. B. Proc. Soc. Exptl. Biol. Med. **66**, 33 (1947).
2. Ware, A. G., Fahey, J. L., and Seegers, W. H. Am. J. Physiol. **154**, 140 (1948).
4. van Creveld, S. and Paulssen, M. M. P. Lancet, **261** No. 2, 242 (1951).
5. Travis, B. L. and Ferguson, J. H. J. Clin. Invest. **30**, 112 (1951).
6. Johnson, S. A., Smathers, W. M., and Schneider, C. S. Am. J. Physiol. **170**, 631 (1952).
7. Deutsch, E. Rev. hématol. **9**, 153 (1954).
8. Deutsch, E., Johnson, S. A., and Seegers, W. H. Circulation Research, **3**, 110 (1955).
9. Horder, M. H. and Sokal, G. Acta Haematol. **14**, 294 (1955).
10. Lewis, J. H. and Ferguson, J. H. Blood, **10**, 351 (1955).
11. Hjort, P., Rapaport, S. I., and Owren, P. A. Blood, **10**, 1139 (1955).
12. Mertz, E. T. and Owen, C. A. Proc. Soc. Exptl. Biol. Med. **43**, 204 (1940).
13. Seegers, W. H., Johnson, S. A., Fell, C., and Alkjaersig, N. Am. J. Physiol. **178**, 1 (1954).
14. Alkjaersig, N., Abe, T., and Seegers, W. H. Am. J. Physiol. **181**, 304 (1955).
15. Quick, A. J. Science, **92**, 113 (1940).
16. Chargaff, E., Moore, D. H., and Bendich, A. J. Biol. Chem. **145**, 593 (1942).
17. Seegers, W. H., McClaughry, R. I., and Fahey, J. L. Blood, **5**, 421 (1950).
18. Seegers, W. H. and Smith, H. P. Am. J. Physiol. **137**, 348 (1942).
19. Ware, A. G. and Seegers, W. H. Am. J. Clin. Pathol. **19**, 471 (1949).
20. Ware, A. G. and Seegers, W. H. J. Biol. Chem. **172**, 699 (1948).

21. WARE, A. G. and SEEGERS, W. H. Am. J. Physiol. **152**, 567 (1948).
22. FOLIN, O. and CIOCALTEAU, V. J. Biol. Chem. **73**, 627 (1927).
23. SEEGERS, W. H. Record Chem. Progr. (Kresge-Hooker Sci. Lib.) **13**, 143 (1952).
24. WARE, A. G. and SEEGERS, W. H. J. Biol. Chem. **174**, 565 (1948).
25. SEEGERS, W. H., LANDABURU, R. H., and FENICHEL, R. L. Am. J. Physiol. **190**, 1 (1957).
26. HECHT, E. R., CHO, M. H., and SEEGERS, W. H. Am. J. Physiol. In press.
27. SEEGERS, W. H. Harvey Lectures, Ser. **17**, 180 (1952–53).
28. SEEGERS, W. H., ALKJAERSIG, N., and JOHNSON, S. A. Am. J. Physiol. **181**, 589 (1955).
29. FUKUTAKE, K., CHO, M. H., and SEEGERS, W. H. Am. J. Physiol. In press.

FURTHER STUDIES ON THE OPTIMUM RATIO OF SATURATED TO MONO-UNSATURATED FATTY ACIDS IN RAT DIETS[1]

T. K. Murray, J. L. Beare, J. A. Campbell, and C. Y. Hopkins

Abstract

Fat mixtures containing 10, 30, 60, and 70% saturated fatty acids as glycerides were fed to male rats at a level of 17% by weight of a chow diet. The saturated acids were mainly palmitic rather than lauric and myristic as previously used. In confirmation of earlier results the fat mixture containing 30% saturated fatty acids produced the greatest weight gains and the highest food efficiency. The same trend was noted when similar fat mixtures were added to a purified diet but differences in weight gain and food efficiency were not significant. The source of fatty acids had no effect on weight gains. The excretion of total lipids was not influenced by the degree of saturation of the fats within the limits studied. Shortening, however, caused increased excretion of total lipid. There were no significant differences in liver and adrenal weights produced by these diets.

In a previous paper, Hopkins, Murray, and Campbell (1) reported that a mixture of vegetable oils containing 30% saturated fatty acids (as glycerides) produced greater weight gains in rats than did mixtures containing a greater or lesser proportion of saturated acids. The hypothesis was advanced that this mixture yielded better weight gains because it was similar in degree of saturation to rat body fat. In that test the saturated fatty acids were supplied mainly by coconut oil and so included much lauric and myristic acids which are normally found in only small amounts in rat body fat. In order to test the general validity of the hypothesis it was necessary to determine if similar results could be obtained with mixtures in which the saturated acids were similar in chain length to those of rat body fat. It was of interest to study the effect of commercial products such as lard, shortening, and corn oil. The effect of changes in the basal diet were also investigated. This paper presents the result of growth studies on these oils along with data on liver and adrenal weights and on total lipid excretion.

Materials and Methods

The basal diet was either a commercial fox chow,[2] containing about 3% fat, or a purified diet the composition of which is shown in Table I. The fats were added to these diets at the rate of 17 g. to 83 g. of chow or 20 g. to 80 g. of purified diet. The fats were bayberry tallow, coconut oil, corn oil, olive oil, vegetable shortening,[3] lard,[4] safflower seed oil, and sunflower seed oil obtained from commercial sources. They were mixed on the basis of their

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Contribution from the Food and Drug Laboratories, Department of National Health and Welfare, Ottawa, and Division of Pure Chemistry, National Research Council, Ottawa.
Issued as N.R.C. No. 4766.
[2]Master Fox Starter Mash, Toronto Elevators Ltd.
[3]Crisco, Procter and Gamble Co. Ltd.
[4]Maple Leaf, Canada Packers Ltd.

TABLE I

COMPOSITION OF PURIFIED DIET

	g./100 g. diet
Casein, vitamin test	19
Corn starch	35
Cerelose	16
Salt mixture, U.S.P. XIV	4
Alphacel	5
Vitamin mixture	1*
Fat	20†

*100 g. vitamin mixture contained: 100 mg. thiamine, 100 mg. riboflavin, 100 mg. pyridoxine HCl, 300 mg. calcium pantothenate, 5 g. inositol, 500 mg. nicotinic acid, 1 g. *p*-aminobenzoic acid, 2 mg. biotin, 0.2 mg. cyanocobalamin, 10 g. choline chloride, 50,000 I.U. vitamin A.
†Fat contained 0.5 mg. menadione, 3000 I.U. vitamin D, and 15 mg. vitamin E per 100 g. diet.

known average composition to give the desired ratio of saturated to unsaturated fatty acids. The composition of the lard was determined by ultraviolet absorption after alkali isomerization (2).

Inbred rats of the Wistar strain were used in all the experiments and were placed on test when they were 5 weeks of age. Animals were divided equally among groups with regard to weight and litter, caged individually, and given food and water ad libitum. A record of food consumption, corrected for wastage, was kept. Feces were collected for a period of 4 days and were frozen until analyzed. Each experiment lasted for 9 weeks. Fecal fat was measured by the method previously described (3).

Results

Effect of Varying the Ratio of Saturated to Mono-Unsaturated Fatty Acids in a Chow Diet

Corn oil, olive oil, and bayberry tallow were mixed in the proportions shown in Table II to give mixtures containing 70, 60, 30, and 10% saturated acids based on the total fatty acids. The content of linoleic acid was 10% in each mixture. Lard with 42% saturated, 46% monoenoic, and 12% dienoic acids was the fifth fat in the comparison. The basal diet was fox chow. There were 20 male rats in each group.

TABLE II

COMPOSITION OF FAT MIXTURES

	Fats, g./100 g.				Fatty acid composition, %		
Diet	Corn	Olive	Bayberry	Lard	Sat'd acid	Oleic acid	Linoleic acid
1	18	14	68	–	70	20	10
2	18	25	57	–	60	30	10
3	14	63	23	–	30	60	10
4	11	89	–	–	10	80	10
5	–	–	–	100	42	46	12*

*May include other dienoic acids.

TABLE III

WEIGHT GAIN, FOOD EATEN, AND FOOD EFFICIENCIES OF RATS FED VARIOUS FAT MIXTURES IN A CHOW DIET

Sat'd acids in dietary fat, %	Weight gain, g.	Food eaten, g.	Weight gain / Food eaten
70	147	656	.224
60	159	680	.224
30	164	663	.244
10	146	618	.240
42 (lard)	160	665	.239
L.S.D.*	9		0.01

*Least significant difference at $P = 0.01$.

After the rats had been 9 weeks on these diets there was a highly significant difference in the weight gains of rats as shown in Table III. On the basis that the weight difference required for significance ($P = 0.01$) was 9 g., the fat containing 30% saturated acids was superior to that containing 10% saturated acids or that containing 70% saturated acids. Both the fat containing 60% saturated acids and the lard (42% saturated) were superior to the 10% and 70% saturated fats.

There was a highly significant difference in the food efficiencies of the various diets. The diets containing 10, 30, and 42% saturated acids were significantly superior to those containing 60% and 70% saturated acids.

Comparison of Fats of Various Degrees of Saturation in Purified and Chow Diets

In order to determine the effect of basal diet on the results, coconut, corn, and olive oil were mixed in the proportions shown in Table IV and added to the purified diet shown in Table I. Two additional groups were fed the same basal diet with added shortening and corn oil. Three of the oils, the 30% saturated mixture, shortening, and corn oil, were also fed with a chow basal diet.

TABLE IV

COMPOSITION OF FAT MIXTURES

Fats, g./100 g.				Composition of mixture		
Coconut	Olive	Corn	Shortening	Sat'd.	Oleic	Linoleic
79	4	17	–	75	15	10
60	26	14	–	60	30	10
23	67	10	–	30	60	10
–	91	9	–	10	80	10
–	–	100	–	13	35	51
–	–	–	100	26	61	12*

*Composition reported by Sreenivasan and Brown (4). "Linoleic" portion consists of 8 parts of true linoleic acid and 4 parts of other dienoic acids.

TABLE V

FOOD EFFICIENCIES, ORGAN WEIGHTS, AND LIPID EXCRETION OF RATS FED VARIOUS FAT MIXTURES IN PURIFIED AND CHOW DIETS

Basal diet	Dietary fat, % sat'd.	Wt. gain, g.	Food efficiency	Liver wt., g.	Adrenal wt., mg.	Neutral fat + fatty acids, g./rat/day	Fatty acids from soaps, g./rat/day
Purified	75	153	0.225	6.25	29.94	0.017	0.022
	60	151	0.226	6.26	26.68	0.024	0.017
	30	164	0.239	6.58	29.73	0.028	0.020
	10	155	0.232	6.50	29.22	0.022	0.019
	Shortening	164	0.234	6.34	29.08	0.026	0.168
	Corn oil	155	0.234	5.77	27.40	0.034	0.023
Chow	30	150	0.210	5.88	26.94	0.082	0.086
	Shortening	141	0.200	6.80	29.57	0.179	0.204
	Corn oil	140	0.202	5.69	27.00	0.081	0.079

The weight gains, food efficiencies, liver weights, and adrenal weights are shown in Table V. While differences between treatments were not significant at $P = 0.05$, it is interesting to note that, of the mixtures, that containing 30% saturated acids gave the highest mean weight gain and the greatest food efficiency. The gains on corn oil and shortening were identical with the mixture of corresponding saturation, i.e. 10% and 30% saturated acids respectively. With the chow basal diet the 30% saturated fat mixture produced the greatest gains but again the differences were not significant.

No significant differences were observed between any of the treatments in liver and adrenal weight.

Table V also shows the results of fecal lipid analyses. When the various fats were added to the purified basal diet there was little difference in the excretion of lipid except for a large soap excretion from rats fed shortening. The excretion of lipid on the chow diet showed the same trend between oils as on the purified diet.

The Effect of the Source of Unsaturated Fatty Acids in a Chow Diet

Fat mixtures containing 30% saturated fatty acids, 27% oleic, and 43% linoleic were prepared as shown in Table VI. These mixtures were added to chow and fed to 20 male rats. It will be noted that in order that large amounts of safflower, cottonseed, and sunflower oil could be used, it was necessary to increase the linoleic acid to 43%.

The weight gains, food consumed, and food efficiencies in Table VI show that the source of the unsaturated fatty acids had no influence on the results.

TABLE VI

COMPARISON OF DIFFERENT MIXTURES OF UNSATURATED ACIDS

Diet	Fats, g./100 g.					Weight gain, g.	Food efficiency
	Coconut	Safflower	Cottonseed	Sunflower	Olive		
1	24	52	–	–	24	121	0.187
2	8	–	82	–	10	124	0.191
3	23	–	–	63	14	122	0.190

Discussion

The superiority of a dietary fat mixture containing 30% saturated fatty acids (as glycerides) over similar fats containing 10% or 70% saturated acids has been confirmed by the present results. The criterion was the weight gain of young male rats and the basal diet was chow. The fact that the chief saturated acid was palmitic, instead of lauric acid as in the previous work (1), has not altered the results. Thus the chain length of the saturated acids, within the limits C_{12}–C_{16}, did not affect weight gain. The linoleate content of the fats was 10% throughout.

It may be concluded that at this level of linoleate there is an optimal ratio of saturated to mono-unsaturated acids in the dietary fat. Stated in another way, this would mean that a certain proportion of saturated acids, as glycerides, is needed in the dietary fat to produce maximum weight gain. The proportion may be in the neighborhood of 30% of the total acids but is at least more than 10% and less than 70%. Lard, with a saturated acid content of 42%, gave about the same weight gain as the fat with 30% saturated acids.

While differences in weight gain were significant in the first experiment using 20 rats on each diet, the results of the second test, based on 10 rats per group and using a purified basal diet, indicated the superiority of the fat containing 30% saturated acids but the differences were not statistically significant. Thus the effect of varying the fatty acid composition, although reproducible, is not large within the limits studied. When various oils were used as sources of unsaturated fatty acids no differences in weight gain were noted.

Analysis of the fecal lipids did not show any significant differences (Table V) when the proportion of saturated acids in the dietary fat was varied from 10% to 75% of the total acids. The possibility cannot be excluded, however, that differences caused by increased saturation of the mixtures may have been masked by the corresponding decrease in average chain length. When corn oil, shortening, and the 30% saturated mixture were compared the shortening diet gave the highest excretion of total lipid. This was largely accounted for by the fatty acids from soaps, probably resulting from incomplete absorption of the stearic acid of the shortening. The total excreted lipid was quite low, however, in relation to the amount of ingested fat in all cases.

No significant differences were observed in the weight of the liver and adrenals of rats fed various fat mixtures.

References

1. Hopkins, C. Y., Murray, T. K., and Campbell, J. A. Can. J. Biochem. and Physiol. **33**, 1047 (1955).
2. Brice, B. A., Swain, M. L., Herb, S. F., Nichols, P. L., Jr., and Riemenschneider, R. W. J. Am. Oil Chemists' Soc. **29**, 279 (1952).
3. Murray, T. K., Campbell, J. A., Chisholm, M. J., and Hopkins, C. Y. J. Am. Oil Chemists' Soc. **35**, 156 (1958).
4. Sreenivasan, B. and Brown, J. B. J. Am. Oil Chemists' Soc. **33**, 86 (1955).

INFLUENCE OF THIAMINE AND TRYPTOPHAN UPON THE TRYPTOPHAN PEROXIDASE–OXIDASE ACTIVITY OF RAT LIVER[1]

EDITH E. TOWNSEND AND T. L. SOURKES

Abstract

The possible role of thiamine in the oxidation of tryptophan by tryptophan peroxidase–oxidase (TPO) of liver was studied by several approaches. (1) In acute experiments parenterally administered thiamine did not cause an adaptive increase in the enzyme content of rat liver, as can be induced by similar injection of tryptophan. (2) Thiamine pyrophosphate, added to liver extracts which had been partially inactivated with respect to TPO by aging for one-half hour at 37° C. or by prolonged dialysis, did not reactivate the enzyme oxidizing tryptophan. (3) In multifactorial dietary experiments in which thiamine, tryptophan, or both were omitted from the diet only tryptophan deficiency resulted in a decrease of the liver TPO activity. There was no effect of thiamine, either in the presence or absence of tryptophan. The D- and L-isomers of tryptophan were equally effective in promoting TPO activity. These results do not support the findings of other authors who imply a role for thiamine in the oxidation of tryptophan by TPO.

Tryptophan peroxidase–oxidase (TPO), an enzyme found in mammalian liver, oxidizes L-tryptophan to N′-formyl-L-kynurenine (1), the first step on one of the "main lines" of tryptophan metabolism. Relatively little is known about the mechanism of action of this enzyme, although there is some evidence pointing to a role for thiamine at some point in the reaction. For example Furuta (2) found that the liver system forming kynurenine from tryptophan is reduced in activity in rats which have been administered the antimetabolite oxythiamine. More recently, Dalgliesh has shown that rats on thiamine-deficient diets exhibit a metabolic block at the level of TPO action (3, 4) as revealed by the pattern of excretion of tryptophan metabolites in the urine of these animals. No explanation for a role of thiamine in the TPO reaction has been available and it was therefore considered worthwhile to investigate this phenomenon further.

TPO has been tentatively classed as an adaptive enzyme (5) because of its rapid increase in the liver of animals shortly after they are administered its substrate, tryptophan (1, 6). In microorganisms adaptive enzymes are lost after the substrate is omitted from the medium. Although such an experimental technique has its obvious limitations in the multicellular organism because of the continuing presence of tryptophan in the body fluids as a result of the breakdown of body protein, it was decided to attempt an approach to this condition by maintaining rats for varying periods of time on a tryptophan-free diet. TPO activity in livers of these animals was compared with the enzyme activity in controls given tryptophan in their diet.

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Contribution from the Allan Memorial Institute of Psychiatry, McGill University, Montreal, Que. This work was supported by a Federal–Provincial Mental Health Research Grant to T.L.S., and by a grant from the Foundations' Fund for Research in Psychiatry, New Haven, to Dr. R. A. Cleghorn. A preliminary account of this work has been presented at the 21st Annual Meeting of the Canadian Physiological Society, Ottawa, October 1957.

Rats are able to utilize D-tryptophan for growth (7) and for the formation of kynurenine (8). In acute tests it is considerably less toxic to rats (intraperitoneal injection) than is the natural isomer (9). Indeed, it may possess whatever toxicity it has by virtue of inversion in vivo to L-tryptophan, as is thought to occur in the other functions mentioned, and if this is the case the rate of inversion could represent a limiting factor in its toxic action. It was considered possible to apply a further test for this isomerization of D-tryptophan in vivo by determining its activity, when supplied in the diet, in stimulating the formation or maintenance of the concentration of liver TPO.

Material and Methods

Animals

Male rats of the Sprague–Dawley strain were used in this work. In the dietary experiments animals were selected for body weight in the range 78 to 130 g. In the acute experiments, larger rats of matched body weight were used.

Diets

Animals were maintained on Purina chow diet until they were ready to be placed on the experimental diets. These were composed for the first nutritional experiment as shown in Table I. The percentage of tryptophan in the diet was altered in various experiments; where it was omitted it was replaced by an equivalent weight of sucrose. Other omissions or alterations are mentioned in the descriptions of the respective experiments.

TPO Activity

The enzyme was assayed as previously described (10). Results are expressed as concentration of enzyme, i.e. μM. of kynurenine formed/hr./g. liver.

TABLE I

COMPOSITION OF EXPERIMENTAL DIETS

Acid-hydrolyzed casein*	19.1%
DL-Phenylalanine	0.45
L-Tyrosine	0.45
Tryptophan (see text)	0.9
Vegetable shortening (Crisco)	13.0
Cod liver oil, U.S.P.	5.0
Salt mixture, U.S.P. XIV	4.0
Cellulose powder	2.0
Sucrose, finely ground	51.1
Vitamin mixture†	4.0
	100.0%

*This material contains no tryptophan, less than 0.1% phenylalanine, and probably less than 0.1% tyrosine.

†The vitamin mixture consisted of the following items: thiamine, 75 mg. (see text); riboflavin, 75 mg.; nicotinic acid, 500 mg.; pyridoxine, 75 mg.; calcium pantothenate, 200 mg.; biotin, 3 mg.; folic acid, 15 mg.; inositol, 5 g.; *p*-aminobenzoic acid, 1 g.; choline, 2 g.; vitamin B_{12} triturate in mannitol, 5 mg.; menadione, 10 mg. These were thoroughly mixed with 91 g. sucrose.

Results

Enzyme Experiments with Thiamine

Two types of experiment were carried out in order to determine whether thiamine has any direct influence on TPO. Subcutaneous injections of thiamine (2 mg./rat) and of L-tryptophan (0.25 mM./rat) in saline were made 3 hours before the rats were sacrificed to assay the liver for TPO activity. A control animal was injected with isotonic saline alone. The relative TPO activity in the livers of the four animals so tested was as follows:

control rat	100%	injected with L-tryptophan	352%
injected with thiamine	96	injected with both	173.

This result indicated that thiamine has no stimulating effect on the adaptive response as do tryptophan and certain other compounds (6, 11). It did not exclude the possibility that thiamine activates the TPO system in vitro when it is added as thiamine pyrophosphate (ThPP). Accordingly this coenzyme was included in reaction mixtures, alone as well as with L-tryptophan as substrate. In Expt. A, Table II, it is seen that the coenzyme had no effect on the oxidation of tryptophan. In Expt. B, it is seen that the coenzyme had no reactivating effect on the aged incubation mixture. Further experiments were done to see whether ThPP restores the activity lost by TPO when liver extracts are dialyzed (1). The results of Expt. C, Table II, show that ThPP

TABLE II

EFFECT OF THIAMINE PYROPHOSPHATE (ThPP) ON TRYPTOPHAN PEROXIDASE–OXIDASE (TPO) IN VITRO

Expt.	Addition	TPO activity
A	Tryptophan, added to enzyme at zero time	1.11
	ThPP added at 0 min.	0.01
	Both, added at 0 min.	1.06
B	Tryptophan, added after enzyme had been incubated 30 min. at 37°	0.40
	ThPP, added at 30 min.	0.00
	Both, added at 30 min.	0.40
C	Dialyzed liver extract	
	Tryptophan	0.76
	Tryptophan+ThPP	0.46
	Non-dialyzed liver extract	
	Tryptophan	2.73
	Tryptophan+ThPP	2.28

NOTE: The enzyme was contained in the supernatant fraction of 25% isotonic KCl rat liver homogenates. The supernatant was obtained by centrifuging the homogenates at 2° for 25 min. at 20,000 × *g*, and was used immediately except in experiments where it was to be dialyzed. Enzyme preparations used in Expt. C were dialyzed for 22 hr. at 2° against 0.0002 *M* L-tryptophan in isotonic KCl. Dialysis against the substrate provided a small degree of protection against the inactivating processes. Reaction mixtures used contained, in a final volume of 2.0 ml.: supernatant fraction 1 ml.; phosphate buffer, pH 7.4, 0.02 *M*; L-tryptophan, 0.002 *M*. Reaction mixtures were incubated at 37° for 1 hr. and were then deproteinized (10% trichloroacetic acid) and centrifuged. Kynurenine resulting from the successive action of TPO and a formylase (the latter present in excess) was measured as diazotizable amine (1). Activity is expressed as μM. of kynurenine formed/hr./g. liver. The data for all experiments have been corrected where necessary for diazotizable substances present in the liver extracts and in Expt. C for kynurenine formed from the residual tryptophan of the dialyzing medium.

had no such action on a liver preparation dialyzed for 22 hours. An even more extensive dialysis of 43 hours against 0.9% KCl containing 0.6% Versene inactivated the TPO preparation and activity was not regained in the presence of magnesium ions or ThPP, or both. The liver extract kept at 2° C. for the same length of time, but not dialyzed, showed considerable TPO activity.

Dietary Experiments

A. Effect of Thiamine and Tryptophan Deficiencies

Because the enzymatic experiments did not reveal an action of thiamine upon TPO we decided to test the activity of the liver extracts in rats which had been deprived of thiamine, having in mind the possibility that the vitamin is required for some indirect action not previously recognized. The effects of tryptophan deficiency on TPO activity were studied simultaneously in a multifactorial experiment, replicated on two occasions. In each replicate three rats were assigned at random to each of the five diets. Hence, a total of 30 rats were used (=3 rats/group×5 diets×2 replicates). The diets were based upon the formula in Table I, with the following variations:

Diet I. As shown in Table I. Supplements of 0.9% DL-tryptophan and thiamine as indicated in the table.

Diet II. Purina checkers, powdered, to serve as a dietary control on the "complete" experimental diet (No. 1).

Diet III. As for Diet I, but omitting thiamine.

Diet IV. As for Diet I, but omitting tryptophan.

Diet V. As for Diet I, but omitting both thiamine and tryptophan.

Sets of rats (i.e. one animal from each dietary group) were sacrificed at intervals and the TPO activity of the livers was determined. In this way data were obtained for a total of six sets of rats sacrificed, respectively, on the 12th, 13th (two sets), 14th, 24th, and 28th days of the experimental diets. It was noted that the rats on the Purina chow diet grew faster than those on Diet I, although the latter gained weight steadily during the experiment. At least two factors could have produced this result: the casein hydrolyzate may not have been satisfactorily completed by the addition of phenylalanine, tyrosine, and tryptophan, so that the mixed protein in the commercial diet offered nutritional advantages. Secondly, it was noticed that rats on Diet I ate much less than those on Diet II, so that palatability may have played a role here. These differences in growth have been delineated for the second replicate in Fig. 1. This graph shows that the tryptophan-deficient rats ceased growing almost at once, and then began to lose weight progressively. The thiamine-deficient rats (Diet III) levelled off in body weight after about 15 days, demonstrating the effect of the avitaminosis.

The data of Table III present the average values, by dietary groups, for each of the parameters measured: change in body weight, liver weight, and the observed TPO activity. The experimental diets thus led to marked differences in body weight and liver weight; in order to account for any effect

independent of the quality of the diets, which such changes may have had on the TPO activity an analysis of covariance was carried out as described by Snedecor (12). The results of this analysis (Table IV) indicate that the differences in TPO activity as a result of the different diets were statistically significant. Because the regression of TPO activity on liver weight and change in body weight was also significant, it was possible to adjust statistically the

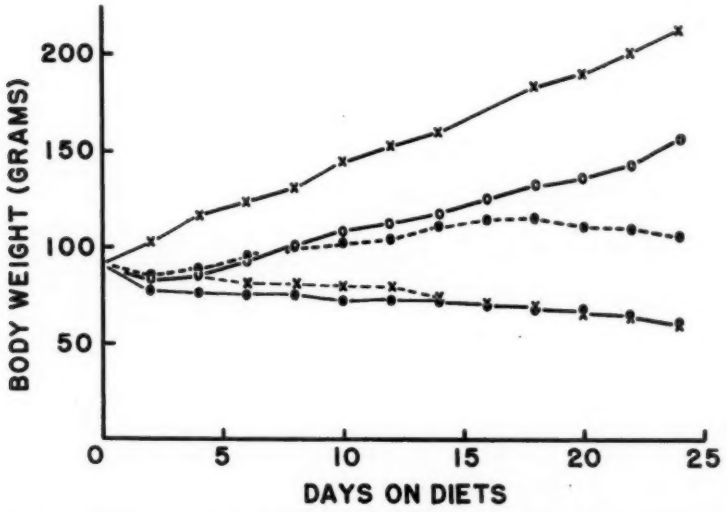


FIG. 1. Growth curves of rats on experimental diets. Each point represents the mean weight of three animals (to 12th day) or two animals (after the 12th day).
×———× rats fed Purina chow;
○———○ rats fed diets supplemented with tryptophan and thiamine;
●------● rats fed diets deficient in thiamine;
×------× rats fed diets deficient in thiamine and tryptophan;
●———● rats fed diets deficient in tryptophan.

TABLE III

EFFECT OF THIAMINE AND TRYPTOPHAN DEFICIENCIES ON BODY WEIGHT, LIVER WEIGHT, AND TPO ACTIVITY OF THE LIVER

Supplement	Change in body weight, g.	Liver weight, g.	TPO activity (μM. kynurenine/hr./g. liver)	
			Observed	Adjusted
Thiamine plus tryptophan ("Complete")	40.2	7.5	2.97	3.04
Tryptophan only	17.7	5.6	3.21	3.22
Thiamine only	−27.2	3.2	1.39	1.42
None	−25.7	3.3	1.44	1.47
Purina chow diet	93.8	8.8	1.94	1.81

NOTE: Each figure is a mean obtained from six rats. See text for composition of the diets. For TPO activity see footnote to Table II. Adjusted values of TPO activity were calculated by use of the respective regression coefficients of liver weight and body weight change on TPO activity, estimated by standard statistical procedure (12).

mean values of TPO activity on each of the diets in order to exclude the effect of these two independent variables. The adjusted mean differences are given in Table III where it is seen that the adjustments are not very marked in spite of the significant regression. The difference necessary for significance at the 5% level of probability, calculated from the residual variance in Table IV is 0.78 μM. of kynurenine/hr./g. liver. This renders the values for the tryptophan-containing synthetic diets significantly greater than the other three. There was no effect of thiamine deficiency, nor was there an interaction of the two nutrients studied.

TABLE IV

ANALYSIS OF COVARIANCE OF DATA ON CHANGE IN BODY WEIGHT, LIVER WEIGHT, AND TPO ACTIVITY OF LIVER

Sources of variation	Degrees of freedom	Mean square	*F* ratio
All	29		
Diets	4	4.347,845	11.762*
Replicates	5	5.503,981	14.889*
Remainder	20	0.475,893	–
Regression of TPO activity on body weight change and liver weight	2	1.432,029	3.874**
Error†	18	0.369,656	(1.0)

*Highly significant (*P* less than 0.01).
**Significant (*P* less than 0.05).
†This variance has been employed to calculate the difference necessary to establish significance between any two mean values of TPO activity each based upon six rats, as given in Table III ("Adjusted Means"). The formula used is

$$\text{Necessary difference} = t\sqrt{(2 \times \text{mean square})/6}.$$

For $2(n-1) = 10$ degrees of freedom, and $P = 0.05$, $t = 2.228$. Hence the minimal difference necessary for significance = 0.78 μM. kynurenine/hr./g. liver.

B. Effect of D- *and* L-*Tryptophan*

In order to study the effect of D-tryptophan on the TPO activity of the liver, two experiments were carried out using diets based upon the one described in Table I. Diets were made up to contain, respectively, D-tryptophan, L-tryptophan, and no tryptophan. In the first experiment the tryptophans were supplied at the level of 0.2%, and in the second they made up 0.4% of the diet. In connection with other work in progress in this laboratory the animals were divided into two major groups, homogeneous with respect to other treatments; one of the groups was injected with reserpine, the other with the solvent medium used to solubilize the alkaloid. Reserpine had no effect on TPO activity (Table V) and its use will not be further considered here. The animals were maintained on the diets for 21 to 26 days after which they were sacrificed to assay the liver TPO. The results (Table V) showed that the D-isomer was as efficient as the L-isomer in promoting enzyme activity. Moreover enzyme activity was considerably reduced in the animals fed the basal diet without a supplement of tryptophan.

TABLE V

EFFECT OF RESERPINE, D-TRYPTOPHAN, AND L-TRYPTOPHAN ON RAT LIVER TPO

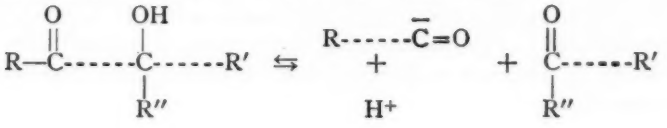
Reserpine	Supplement*	No. of rats	Change in body wt., g.	Liver weight, g.	TPO activity†
−	None	7	−18.8	2.9	2.46
	D-Tryptophan	7	− 2.8	3.7	5.09
	L-Tryptophan	7	− 4.7	3.8	5.98
+	None	7	−23.0	2.9	2.53
	D-Tryptophan	6	− 2.4	3.4	6.59
	L-Tryptophan	7	− 5.0	3.4	5.47
Standard deviation of means‡					
(*a*) Of 6 rats			± 2.8	±0.19	±0.64
(*b*) Of 7 rats			± 2.6	±0.17	±0.59

*See text for description of basal diet.
†Footnote to Table II.
‡Calculated from the mean square for error obtained in the analysis of variance, after removal of variance between the diets and between the replicates. These statistical analyses have been omitted to conserve space; they showed that the animals receiving the tryptophan-free diet were inferior to the supplemented animals in respect to body weight, liver weight, and TPO activity (with a probability of 0.05 or less), as is evident upon inspection of the above summary table.

Discussion

Until recently thiamine in its coenzyme form was known to be involved in but one type of reaction, namely the decarboxylation of pyruvic acid to form acetaldehyde and carbon dioxide (13, 14, 15). Its role in the oxidative decarboxylation of the α-keto acids was elucidated with the discovery of thioctic acid (16). Shortly afterwards thiamine pyrophosphate (ThPP) was found to be the coenzyme for transketolation also (17, 18), as in the formation of sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate by a reaction between the 5-phosphates of ribose and ribulose. The transferring, in contradistinction to the decarboxylative, function was consequently considered an unusually novel role for ThPP indicating a broader scope for its participation in intermediary reactions than had been hitherto suspected. A third reaction in which ThPP has been presumed to play a role is the oxidation of tryptophan to N′-formyl-L-kynurenine (3, 4). The function of thiamine in this conversion has been demonstrated thus far only by a technique employing intact animals. The carboxylase and transketolase reactions, on the other hand, have been performed with relatively purified materials which require the addition of ThPP for activity. The differences between these reactions, particularly between the latter two, which have been delimited by the methods of enzyme chemistry, may, however, belie a fundamentally similar activity.

Symbolically these reactions may be represented as follows:



For decarboxylation of pyruvic acid, R = CH_3, and R′R″ = O; for the C_2—C_3 scission of ribulose-5-phosphate, R = CH_2OH, R′ = H, and R″ = $CHOH.CH_2O.PO_3H_2$. In this transketolase reaction molecular glycolaldehyde is not actually formed; instead the elements of this compound are transferred in a ketol type of condensation to ribose-5-phosphate. Hydroxypyruvic acid (18) and L-erythrulose (17), which can also undergo this reaction, easily fit into the representation shown. The oxidation of tryptophan to formylkynurenine is initiated by a specific bifunctional enzyme, the peroxidase–oxidase (19). In this oxidation there occurs the rupture of a carbon–carbon linkage in the indole ring. The probable sequence of events, as suggested by Knox (19) is the addition of hydrogen peroxide to tryptophan, followed by oxidation of the resulting compound with the formation of the ketol, β-(2-oxo-3-hydroxy-3-indolyl)-alanine, and H_2O_2. Formylkynurenine, a structural isomer of this ketol, results from a reaction quite analogous to the others described above. In this case R = —$NH.C_6H_4$— (the substituents of the benzene ring being ortho to one another), R′ is the inverse of R, i.e. —$C_6H_4.NH$—, and R″ is the alanine side chain. This mechanism of oxidation of tryptophan is not concurred in by Ek and his colleagues (20), but it satisfies the requirements of the enzymatic data and agrees with what is known about other ThPP-linked reactions. Whether this mechanism is actually operative in the TPO reaction or not awaits the result of further experiments to define the role of thiamine therein.

The data reported in the present work do not provide support for Dalgliesh's finding implicating thiamine in the initial stages of tryptophan breakdown. Similarly negative results were obtained by Ginoulhiac (21), whose report came to our attention while this work was in progress. Indeed, Ginoulhiac found a tendency for the TPO activity to increase in the livers of thiamine-deficient rats, a tendency which was also present in our experiments (Table III), although not very marked.

The possibility exists, of course, that thiamine is nevertheless involved in TPO activity but that the apoenzyme binds it very tenaciously. In such a case activity might be retained under the most adverse conditions of thiamine deficiency. Judging from the weight curves presented here the animals were being depleted of their tissue thiamine, although none of them displayed signs of polyneuritis as did rats in Ginoulhiac's experiments (21). There was no evidence that ThPP reactivates TPO after the liver extracts containing it had been dialyzed for either 22 or 43 hours (Table II).

The D- and L-isomers of tryptophan were found to be equally efficient in promoting TPO activity as evidenced in experiments lasting 3 to 4 weeks. This is in agreement with other work demonstrating the ability of the rat to utilize D-tryptophan for growth (7), with incorporation of tryptophan into tissue proteins (22). The mechanism of the inversion of D-tryptophan to the natural isomer is not known but it may involve deamination followed by stereospecific reamination (23).

Finally, it has been shown that deprivation of tryptophan results in significant reductions in the TPO activity compared to the levels observed in livers of tryptophan-supplemented rats. TPO activity is responsive not only to tryptophan but also to histidine, tyrosine (6, 11), and perhaps other compounds, and it is likely that these are present in sufficient concentration in the tissues of the deficient animals, through breakdown of tissue protein, to maintain some activity of the enzyme. The relatively low activity in the livers of rats fed Purina chow (a "natural" diet) as compared with animals on the synthetic diet may be due to a lower concentration of tryptophan in the former, lower availability of the amino acid when offered in protein form, or both.

Acknowledgments

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References

1. KNOX, W. E. and MEHLER, A. H. J. Biol. Chem. **187**, 419 (1950).
2. FURUTA, K. Ôsaka Daigaku Igaku Zassi, **8**, 131 (1956); *seen in* Chem. Abstracts, **50**, 11459*f* (1956).
3. DALGLIESH, C. E. Biochem. J. **61**, 328 (1955).
4. DALGLIESH, C. E. Brit. Med. Bull. **12**, 49 (1956).
5. KNOX, W. E., AUERBACH, V. H., and LIN, E. G. C. Physiol. Revs. **36**, 164 (1956).
6. KNOX, W. E. and MEHLER, A. H. Science, **113**, 237 (1951).
7. DU VIGNEAUD, V., SEALOCK, R. R., and VAN ETTEN, C. J. Biol, Chem. **98**, 565 (1932).
8. BORCHERS, R., BERG, C. P., and WHITMAN, N. E. J. Biol. Chem. **145**, 657 (1942).
9. GUILLINO, P., WINITZ, M., BIRNBAUM, S. M., CORNFIELD, J., OTEY, M. C., and GREENSTEIN, J. P. Arch. Biochem. Biophys. **64**, 319 (1956).
10. SOURKES, T. L. and TOWNSEND, E. Can. J. Biochem. and Physiol. **33**, 735 (1955).
11. KNOX, W. E. Brit. J. Exptl. Pathol. **32**, 462 (1951).
12. SNEDECOR, G. W. Statistical methods. 4th ed. Iowa State College Press, Ames, Iowa. 1946. Chap. 10.
13. GREEN, D. E., HERBERT, D., and SUBRAHMANYAN, V. J. Biol. Chem. **138**, 327 (1941).
14. LOHMANN, K. and SCHUSTER, P. Biochem. Z. **294**, 188 (1937).
15. KUBOWITZ, F. and LÜTTGENS, W. Biochem. Z. **307**, 170 (1941).
16. REED, L. J. Physiol. Revs. **33**, 544 (1953).
17. HORECKER, B. L. and SMYRNIOTIS, P. Z. J. Am. Chem. Soc. **75**, 1009 (1953).
18. RACKER, E., DE LA HABA, G., and LEDER, I. G. J. Am. Chem. Soc. **75**, 1010 (1953).
19. KNOX, W. E. Biochim. et Biophys. Acta, **14**, 117 (1954).
20. EK, A., KISSMAN, H., PATRICK, J. P., and WITKOP, B. Experientia, **8**, 36 (1952).
21. GINOULHIAC, E. Boll. soc. ital. biol. sper. **32**, 1178 (1956).
22. SCHAYER, R. W. J. Biol. Chem. **187**, 777 (1950).
23. MEISTER, A. H. Biochemistry of the amino acids. Academic Press, Inc., New York. 1957. p. 110.

ON THE MECHANISM OF GLUCOSE-6-PHOSPHATE OXIDATION IN CELL-FREE EXTRACTS OF XANTHOMONAS PHASEOLI (XP8)[1]

R. M. Hochster and H. Katznelson

Abstract

Extracts of the plant pathogenic organism *Xanthomonas phaseoli* contain both TPN- and DPN-linked glucose-6-phosphate dehydrogenases as well as a DPN-specific glyceraldehyde phosphate dehydrogenase. DPNH oxidase is very active and sensitive to high concentrations of cyanide. No evidence could be found for the presence of pyridine nucleotide transhydrogenase.

Ribose-5-phosphate is metabolized via the pentose cycle; sedoheptulose phosphate is an intermediate and other criteria of the functioning of the pentose cycle are met when ribose-5-phosphate is the substrate. The same system also functions with 6-phosphogluconate as substrate but at a level which suggests that this pathway is not of great significance.

The Entner–Doudoroff 6-phosphogluconate-splitting pathway is the major avenue of hexosephosphate utilization in cell-free extracts. Pyruvate formation and utilization are discussed in relation to function and cofactor requirements.

While phosphoglucose isomerase, aldolase, triosephosphate isomerase, and the enzymes converting phosphoglyceraldehyde to pyruvate are present and enzymatically active, phosphofructokinase appears to be the "bottleneck" to glycolysis. On the other hand fructose-1,6-diphosphate is oxidized by both DPN- and TPN-linked pathways, the latter through the efficient functioning of a specific phosphatase which forms fructose-6-phosphate readily. Glycolysis is considered to play only a very minor role in these extracts.

A new "hexose cycle" for the metabolism of hexosephosphate is proposed. It consists of oxidation through "Zwischenferment", followed by the Entner–Doudoroff splitting system and resynthesis of hexosemonophosphate through reversal of the aldolase reaction followed by specific carbon-1 phosphate hydrolysis of fructose-1,6-diphosphate. The cycle is shown to be metabolically feasible and thermodynamically likely. It is considered to be the pathway by which cells which tend not to utilize the pentose cycle or classical glycolysis, but have an active 6-phosphogluconate-splitting system, metabolize hexosephosphate. Pyruvate produced as a result of the operation of this system is then oxidized via the tricarboxylic acid cycle in glucose-grown cells.

Introduction

The concept of the existence in all living cells of alternate pathways for the oxidation and utilization of carbohydrate is now well established experimentally. As a consequence to the development of at least three of these pathways (glycolysis, direct hexosemonophosphate oxidation via the pentose cycle, and the direct splitting reaction of 6-phosphogluconate) glucose-6-phosphate emerges as a pivotal intermediate strategically located between the two chief alternative routes: glycolysis and direct hexosemonophosphate oxidation. Factors which, under physiological conditions, control the direction in which oxidation proceeds are as yet not known.

A program has recently been initiated in our laboratories designed to help unravel the complex problem of the mechanism of invasion of the plant host by phytopathogenic bacteria through a thorough study of the metabolism of these organisms. Previous investigations on the metabolism of plant patho-

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genic bacteria include: a comparative study on some general aspects of carbohydrate metabolism by the major genera *Xanthomonas*, *Pseudomonas*, *Agrobacterium*, *Erwinia*, and *Corynebacterium* (1, 2), observations on the formation of pyruvate by these genera (3), studies on the catabolism of hexuronic acids by *Erwinia carotovora* (4, 5), and preliminary notes on the oxidative metabolism by *Corynebacterium insidiosum* (6). As yet no detailed study has been recorded of the metabolic interrelationships existing in any single phytopathogenic organism. This paper is concerned specifically with the mechanism of glucose-6-phosphate oxidation by extracts of *Xanthomonas phaseoli* (XP8), the causal agent of common blight of beans.

Materials and Methods

Materials

The following substances used in this investigation were commercial preparations: ADP,* ATP, DPN, TPN, and CoA (Pabst Brewing Co.); G-6-P, F-6-P, FDP, PGA, GSH, TTC, Polidase, and lithium pyruvate (Schwarz Laboratories); tris, 6-PG (see also note below), lipoic acid, α-ketoglutaric acid, DPNH, TPNH, (Sigma Chemical Co.); R-5-P, ThPP, *p*CMB, dihydroxyacetone, glycylglycine, isocitric acid, malic acid, and fumaric acid (Nutritional Biochemicals Corp.); potassium gluconate (Chas. Pfizer & Co.); and iodide-free sodium IOA and sodium succinate (Eastman Kodak Co.).

6-PG was also prepared in our own laboratory by a modification of the method of Seegmiller and Horecker (7). When this or the commercial product was tested for purity by chromatographic and ionophoretic means (8) it was found that, provided the sodium salt of 6-PG was used immediately following its conversion from the barium salt, it consisted of a single component. However, if the product was left to stand, even at 0–4° C. at neutral pH, other substances began to appear. Thus on the third day three components could be detected which corresponded chromatographically with 6-phosphogluconolactone, G-6-P, and 6-PG. It was, therefore, necessary to use 6-PG always immediately after its preparation from the barium salt in order to obtain unequivocal results.

D-Glyceraldehyde-3-phosphate was obtained through the courtesy of Dr. C. E. Ballou in the form of the dimethyl acetal and converted to the free acid by the method of Ballou and Fischer (9). A sample of sedoheptulosan monohydrate was kindly donated by Dr. J. K. N. Jones. Analytical work with this material was based on calculations of the sedoheptulose content

*The following abbreviations are used: ADP, adenosine diphosphate; ATP, adenosine triphosphate; DPN, diphosphopyridine nucleotide; DPNH, reduced form of diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; TPNH, reduced form of triphosphopyridine nucleotide; CoA, coenzyme A; GSH, reduced glutathione; R-5-P, D-ribose-5-phosphate; Ru-5-P, D-ribulose-5-phosphate; Xu-5-P, D-xylulose-5-phosphate; SH-7-P, sedoheptulose-7-phosphate; G-6-P, D-glucose-6-phosphate; F-6-P, D-fructose-6-phosphate; FDP, D-fructose-1,6-diphosphate; 6-PG, 6-phosphogluconate; KDPG, 2-keto-3-deoxy-6-phosphogluconate; E-4-P, D-erythrose-4-phosphate; GAP, D-glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; PGA, phosphoglyceric acid; PEP, phosphoenol pyruvic acid; IOA, iodoacetate; *p*CMB, parachloromercuribenzoate; ThPP, thiamine pyrophosphate; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane; TTC, triphenyltetrazolium chloride; $MgCl_2$, magnesium chloride.

following prior treatment with acid (10). As an accurate analytical standard a sample of sedoheptulose-*o*-nitrophenylhydrazone kindly supplied by Dr. A. C. Neish was used following hydrazine removal (11). Excellent agreement was obtained between values arrived at by the two methods.

Analytical Methods

Heptulose was identified on paper chromatograms by spraying with TCA–orcinol (12) after the papers were developed with propanol – glacial acetic acid – water 60:1:39 (13). For accurate heptulose determination, an aliquot of solution was chromatographed for 16 hours, its position located by means of reference strips cut from the same papers, eluted from the sheets, and analyzed by the specific method of Dische (14) using "Procedure 2" with heating after acetic acid addition.

The determination of hexose- and triosephosphates in the presence of other sugar phosphates was done as previously described (15). Pyruvate was determined either by the direct or by the double extraction methods of Friedemann and Haugen (16) using toluene as solvent. Triose was measured as a chromogen in presence of 2,4-dinitrophenylhydrazine and alkali (17) and as alkali-labile phosphate. Orthophosphate was determined by the method of King (18).

Protein concentration was estimated by the spectrophotometric method of Warburg and Christian (19).

Enzyme Assays and Measurement of Oxidation

G-6-P, 6-PG, and GAP dehydrogenases were assayed by standard procedures (20, 21, 22) using phosphopyridine nucleotide reduction as a measure of the reaction rate. The reactions were carried out in a Beckman Spectrophotometer (Model DU) at 340 mμ, at 22–23° C. Measurements of the Entner–Doudoroff (23) pathway of 6-PG oxidation were carried out according to the detailed directions published by Kovachevich and Wood (24, 25).

Tests for pyridine nucleotide transhydrogenase in crude extracts were carried out as described by Colowick *et al.* (26) but a more direct method using TPNH as substrate was also applied (see *Experimental*). TTC reduction was measured as described by Kun and Abood (27).

Aerobic oxidation experiments were done in conventional Warburg vessels using KOH papers, air as the gas phase, and a temperature of 30° C. in a total volume of 3.2 ml. Kinase activity was measured by determining anaerobic CO_2 evolution from bicarbonate (28) as well as by other, more indirect means (see *Experimental*). Blanks have been subtracted from all figures given in this paper and the substrate was always tipped in from the side arm. All other incubation experiments were also done at 30° C.

Enzyme Preparations

Cells were grown as described previously (1). They were harvested by centrifugation at 4000 r.p.m., washed twice with 0.9% NaCl, and suspended in distilled water. Each batch of cells was tested for strain purity using the specific phage test (29).

(*a*) *Frozen Cell Extracts*

Ten grams (wet weight) of packed cells were suspended in 13 ml. distilled water and the slurry frozen quickly in acetone – dry ice as a thin film. It was then stored at −28° C. until required. Before use, the frozen cell-paste was allowed to reach room temperature, was diluted with a further 7 ml. of water, and then treated with sonic vibration in a Raytheon 200-watt, 10-kc. oscillator for 20 minutes at a power output of 1.0 amp. The suspension was then centrifuged at 11,000 r.p.m. (15,400×*g*) at 0° C. for 10 minutes and only the uppermost layer was used for the experiments reported here.

(*b*) *Fresh Cell Extracts*

These were prepared from freshly grown (18 hour) cultures, harvested, washed, and treated by sonic oscillation exactly as described above using a dilution of 2 ml. water per 1 g. wet weight of packed cells. It should be noted that in most of the experiments reported in this paper results obtained with extracts of frozen cells were identical with those from freshly harvested cells. The data given are representative of typical experiments. Where differences were obtained between extracts of frozen and fresh cells these are pointed out.

When loopfuls of either extract were plated out on glucose–yeast extract-agar plates and incubated for 3 days at 27° C., no evidence of growth was detectable.

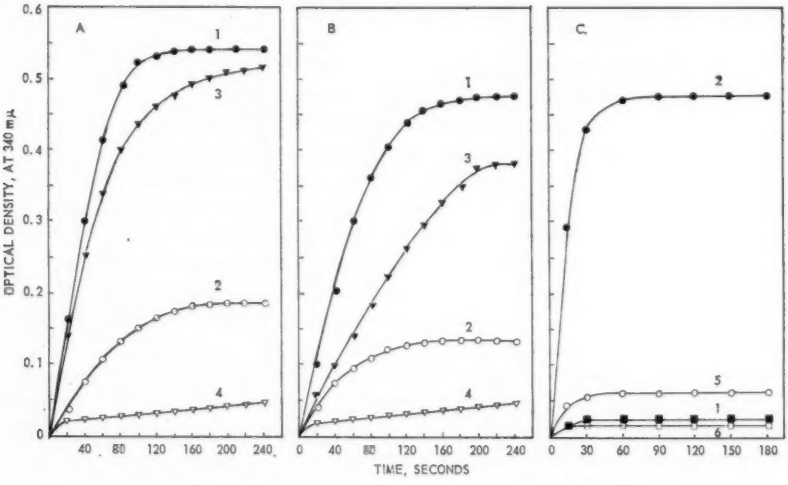

FIG. 1. Spectrophotometric determinations of G-6-P (A), 6-PG (B), and GAP (C) dehydrogenases and their cofactor specificities.

Curve 1 = TPN, 2 = DPN, 3 = DPN + cyanide, 4 = DPN + cyanide (without substrate), 5 = DPN + IOA, 6 = TPN + IOA.

Cuvette contents for experiments A and B:

Substrates, 30 μM.; DPN or TPN, 0.3 μM. (added last, at zero time); glycylglycine buffer pH 7.8, 100 μM.; $MgCl_2$, 10 μM.; sodium cyanide pH 7.8, 150 μM.; cell-free extract, 0.05 ml. (2 mg. protein) in A, 0.15 ml. (6 mg. protein) in B; total volume, 3 ml.

Cuvette contents for experiment C: substrate, 10 μM.; DPN or TPN, 0.3 μM. (added last, at zero time); sodium pyrophosphate buffer pH 8.5, 50 μM.; cysteine, 40 μM.; sodium arsenate, 40 μM.; IOA, 20 μM.; cell-free extract 0.1 ml. (6 mg. protein); total volume, 3 ml.

Experimental and Results

Hexose- and Triose-phosphate Dehydrogenases

When the oxidative metabolism of G-6-P is considered it is at once apparent that the dehydrogenases catalyzing the oxidations of G-6-P to 6-PG and of 6-PG to Ru-5-P are likely to be linked to terminal respiratory systems which may play an important role in the energetics of the cell. Studies on the pyridine nucleotide specificities of these dehydrogenases have shown that TPN is readily reduced (Fig. 1, A and B, curves 1). Unlike the mammalian, yeast, or *E. coli* G-6-P and 6-PG dehydrogenase preparations (30), which show strict TPN-specificity, the *X. phaseoli* extracts also contain DPN-linked dehydrogenases, similar to those in *Pseudomonas fluorescens* (31), *Pseudomonas lindneri* (32), *Leuconostoc mesenteroides* (33), and *Aspergillus flavus-oryzae* (34). The rates of reaction with DPN as cofactor, as shown in Fig. 1 (A and B, curves 2), are much lower than those of the corresponding TPN-catalyzed systems (curves 1), and reach much lower equilibrium values. Furthermore, the addition of high concentrations of cyanide ($5\times10^{-2}M$) to the DPN-catalyzed systems greatly stimulates DPN reduction (Fig. 1, A and B, curves 3). These results suggest the functioning of a cyanide-sensitive competing reaction by means of which DPNH, once formed, is quickly reoxidized. That this is actually the case may be seen from Fig. 2B, in which evidence for the highly active DPNH

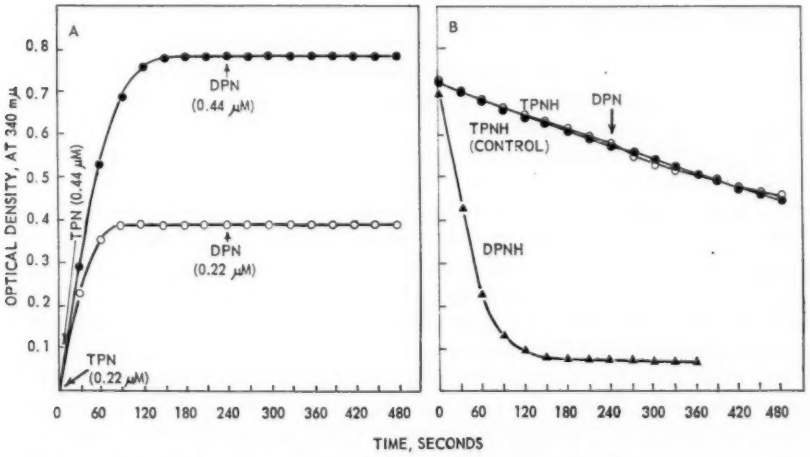

FIG. 2. The activity of DPNH oxidase and some attempts to demonstrate pyridine nucleotide transhydrogenase by two spectrophotometric methods: A, by the method of Colowick *et al.* (26) using excess G-6-P in presence of G-6-P dehydrogenase as the TPNH generating system; B, by a direct method.

Cuvette contents for experiment A: G-6-P, 5 μM.; sodium phosphate buffer pH 7.5, 80 μM.; $MgCl_2$, 10 μM.; cell-free extract, 0.05 ml. (2 mg. protein); total volume, 3 ml. Pyridine nucleotides, quantities and types as shown.

Cuvette contents for experiment B: glycylglycine buffer pH 7.8, 100 μM.; $MgCl_2$, 10 μM.; cell-free extract, 0.075 ml. (3 mg. protein); total volume, 3 ml. Reduced pyridine nucleotides (0.4 μM.) added at zero time, 0.4 μM. DPN added at the time indicated.

TABLE I

SOME INHIBITORS OF DPNH OXIDASE ACTIVITY

System*	Molarity of inhibitor	Net drop in optical density at 340 mμ in first 2 min. of reaction	Inhibition, %
DPNH	—	0.473	—
DPNH + cyanide	1×10^{-3}	0.285	40.0†
DPNH + cyanide	1×10^{-2}	0.050	89.5†
DPNH + cyanide	5×10^{-2}	0.008	98.3†
DPNH + azide	2×10^{-2}	0.439	7.2
DPNH + *p*CMB	2×10^{-3}	0.249	47.4
DPNH + hydroxylamine	2×10^{-2}	0.388	18.0
DPNH + cysteine	1×10^{-2}	0.216	54.4
DPNH + arsenate	1×10^{-2}	0.227	52.0
DPNH + pyrophosphate	3×10^{-2}	0.216	54.4
DPNH + cysteine + arsenate + pyrophosphate	As above	0.080	83.0
DPNH + cysteine + arsenate + pyrophosphate, *but:* without glycylglycine buffer	As above	0.041	91.4

*Components of the common system: glycylglycine buffer, pH 7.7, 100 μM.; $MgCl_2$, 10 μM.; cell-free extract, 0.05 ml. (2 mg. protein); DPNH, 0.4 μM.; total volume, 3 ml.

†These values are uncorrected for the slight contribution made to the value at 340 mμ by the interaction between cyanide and DPN (see Fig. 1, Curves 4A, 4B) at the pH used.

oxidase system is presented, and from Table I in which data for the inhibition of such a system are given. No corrections have been made for the small contribution to the absorption at 340 mμ made by the interaction between cyanide and DPN at this pH; instead, the actual curves are given (Fig. 1, curves 4A and 4B).

The triosephosphate dehydrogenase system in *X. phaseoli* is the conventional DPN-specific type, highly sensitive to iodoacetate (Fig. 1C). It should, perhaps, be mentioned also that addition of similar concentrations of iodoacetate to the G-6-P and 6-PG dehydrogenases had no effect whatsoever. It has also been found that the 6-PG dehydrogenase is occasionally present in extracts in much lower concentration than the G-6-P dehydrogenase. On some occasions as much as a fivefold increase in the amount of enzyme preparation was necessary to obtain an equivalent amount of TPN reduction. This finding may explain an earlier observation (3) suggesting a lack of 6-PG dehydrogenase activity in *Xanthomonas* species.

Reduced Diphosphopyridine Nucleotide Oxidase

Evidence for the presence of this enzyme system has been given in Fig. 2B and results of inhibition studies are presented in Table I. Upon inspection of Fig. 1, one could raise the question as to why it was necessary to add cyanide in order to demonstrate full DPN-catalyzed dehydrogenase activity when G-6-P and 6-PG were the substrates while in the presence of GAP, DPN

reduction took place readily and apparently unhampered by the DPNH oxidase. It was found, however, that cysteine, arsenate, and pyrophosphate each inhibited the DPNH oxidase to the extent of about 50%. Furthermore, when all three components were present together, especially in the absence of glycylglycine buffer, under the exact conditions employed in the assay of GAP dehydrogenase (22), these substances together caused an inhibition of 91.4%. This result fully explains the reason why DPN reduction proceeds almost to completion under the conditions of the GAP dehydrogenase assay.

The oxidation of substrate amounts of DPNH has also been demonstrated by the manometric technique. When 10 μM. of DPNH is tipped from the side arm after a 15-minute equilibration period, approximately 83% of the calculated oxygen uptake for the DPNH added was consumed in the first 10 minutes (calculated on the basis of $\frac{1}{2}$ M. O_2 per mole DPNH oxidized). During the next 50 minutes, the reaction proceeded slowly to completion. Kinetically, this behavior is similar to that described by Eichel (35) for the DPNH oxidase of *Tetrahymena pyriformis*.

It should also be mentioned that triphenyltetrazolium chloride was not reduced by cell-free extracts of *X. phaseoli* under aerobic conditions carried out according to Kun and Abood (27) with DPNH as substrate.

Pyridine Nucleotide Transhydrogenase

Since the discovery of pyridine nucleotide transhydrogenase by Colowick *et al.* (26) it has become necessary to examine nucleotide specificity data with extreme care. This enzyme catalyzes the reaction: TPNH+DPN$\rightleftharpoons$TPN+DPNH. Thus, in its presence, it becomes possible that data attributed to the one nucleotide may in fact be due to the other as a result of their mutual interconvertibility. For the present and future studies it was, therefore, necessary to establish the presence or absence of this enzyme in our extracts.

Experiments were performed in the now classical manner as described by Colowick *et al.* (26) with excess substrate but using G-6-P dehydrogenase as the TPNH generating system. The results are given in Fig. 2A. They show that adequate provision for oxidizable substrate has been made, as seen from the optical densities obtained when 0.22 or 0.44 μM. TPN were used. Under these conditions and in the presence of a transhydrogenase, addition of a similar quantity of DPN should, in presence of excess substrate, give rise to a further, equivalent increase in optical density since more TPN would have been formed. This was not found to be the case. There is, however, an added complication. In the presence of an active DPNH oxidase, it may be argued that the DPNH which could theoretically be formed by such a transhydrogenase would be readily reoxidized back to DPN, thus nullifying any possible increase in optical density.

These objections can be overcome by carrying out a more direct type of experiment as seen in Fig. 2B. It is readily observed that our extracts contain much less TPNH oxidase activity when compared to the highly active DPNH oxidase. Following establishment of a control rate of TPNH oxidation, another cuvette is set up and at the time indicated (arrow) DPN is added.

If there were a transhydrogenase present, addition of DPN would readily cause the formation of DPNH+TPN. But in the presence of the highly active DPNH oxidase as shown here, the DPNH would be immediately re-oxidized, probably at a rate identical to the control DPNH oxidation curve as shown in Fig. 2B. A net drop in optical density equivalent to the amount of DPNH reoxidized should have taken place. The figure shows, however, that DPN addition did not cause a change in rate of TPNH oxidation. Identical results were obtained when comparing data from fresh cell, as against frozen cell extracts. These data are considered proof that pyridine nucleotide transhydrogenase is not present in extracts of *X. phaseoli* studied under our conditions.

Ribose-5-phosphate Oxidation

R-5-P is readily oxidized under aerobic conditions by fresh cell extracts as measured in Warburg manometers (see Table VII). Spectrophotometrically, the results shown in Fig. 3 were obtained. They show that in the presence of R-5-P, DPN is reduced quickly; that arsenate speeds up the initial phases of the reaction; and that this DPN reduction is sensitive to IOA. These facts are consistent with the view that they represent, in fact, determinations of triosephosphate dehydrogenase activity. This is not surprising since a 7-minute incubation period was allowed in these experiments before DPN was

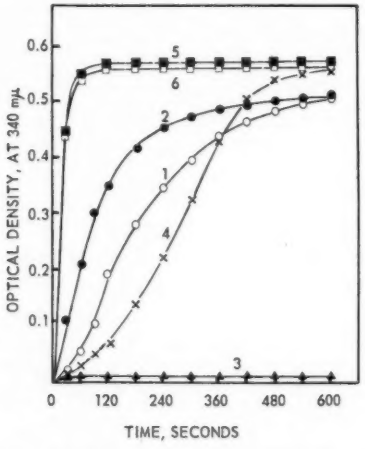

FIG. 3. Oxidation of R-5-P as measured spectrophotometrically by pyridine nucleotide reduction.
1 = DPN only, 2 = DPN + arsenate, 3 = DPN + arsenate + IOA, 4 = TPN (added 10 seconds after enzyme addition), 5 = TPN (added 10 minutes after enzyme addition), 6 = as in 5, plus IOA.

Concentration of components, curves 1–3: R-5-P, 30 μM.; sodium pyrophosphate buffer pH 8.5, 50 μM.; $MgCl_2$, 10 μM.; cysteine, 40 μM.; sodium arsenate, 40 μM.; IOA, 50 μM.; cell-free extract 0.1 ml. (6 mg. protein); DPN (added at zero time), 0.3 μM.; total volume, 3 ml.

Concentration of components, curves 4–6: R-5-P, 30 μM.; glycylglycine buffer pH 7.85, 100 μM.; $MgCl_2$, 10 μM.; IOA, 30 μM.; cell-free extract, 0.1 ml. (6 mg. protein); TPN (added at the times indicated above) 0.3 μM.; total volume, 3 ml.

added, in keeping with conditions for maximum GAP dehydrogenase activity (22). Furthermore, in the presence of cysteine, arsenate, and pyrophosphate buffer, approximately 90% of the DPNH oxidase activity is inhibited (see Table I), thus, the optical density value for the DPNH formed is approximately 10% below the theoretical maximum. The net formation of GAP (the true substrate in the above determination) during the transketolase–transaldolase series of reactions has been demonstrated previously (15, 36).

TPN was also reduced most effectively but only after a short period of incubation with the enzyme preparation (Fig. 3, cf. curves 4 and 5). The lag period shown in the system in which measurements were begun immediately is interpreted as representing the time required for pentose cycle substrate (i.e. G-6-P) formation through the isomerase–epimerase–transketolase–transaldolase series of reactions (37). The insensitivity of the TPN-linked system to IOA is also easily recognizable (Fig. 3, curve 6).

From these data it is suggested that when DPN is used as cofactor in the oxidation of R-5-P, oxidation proceeds via the DPN-specific GAP dehydrogenase to the terminal respiratory carrier chain. When TPN is used, oxidation most probably takes place via the pentose cycle and G-6-P dehydrogenase, a pathway known to be insensitive to IOA in most tissues studied to date. The above experiment provides evidence, although quite indirect, for the functioning of the complete pentose cycle in *X. phaseoli* when R-5-P is the substrate employed. Further evidence for the latter will be given below.

Sedoheptulose Phosphate as an Intermediate

If the complete pentose cycle is, in fact, operating in our extracts it should be possible to demonstrate both the formation and the disappearance of SH-7-P. A series of quantitative experiments was carried out using a combination of incubation, aliquot chromatography, and specific analytical determination as described under *Analytical Methods*. The results, given in Fig. 4, show that SH-7-P is formed rapidly from R-5-P reaching a maximum value of 4.1 μM. (from 10.0 μM. R-5-P) in 5 minutes. An equal amount (4.1 μM.) of GAP should also be present following the transketolase step, although a value obtained after 5 minutes' incubation showed 3.86 μM. triosephosphate as alkali-labile phosphate. From theory: 2 pentosephosphate→1 heptulose phosphate+1 triosephosphate, thus, 5.0 μM. would have been a theoretically attainable maximum of SH-7-P if all the initial pentose had been converted to heptulose+triose. However, the substrates for the transketolase reaction are R-5-P and Xu-5-P (38, 39) with Ru-5-P forming part of the initial equilibrium. The latter compound does not actually take part in the transketolase reaction and it is therefore conceivable that at the 5-minute mark the remaining 0.9 μM. may be in the form of Ru-5-P. That this is probably the case was shown as follows. A sample of the reaction mixture was removed after 5 minutes' incubation and treated with TCA to remove protein. The sample was then neutralized and treated with phosphatase as previously described (13). The remaining free-sugar mixture was then chromatographed, sprayed, and oversprayed (13). Apart from sedoheptulose only a pink spot characteristic

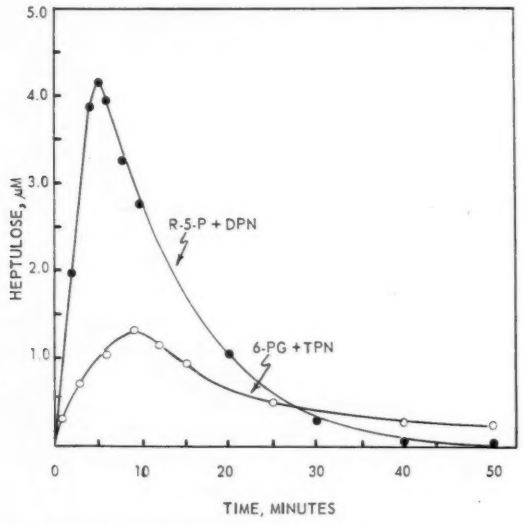

FIG. 4. Heptulose formation and disappearance during aerobic incubation of R-5-P and 6-PG with *X. phaseoli* extracts.

Components of initial reaction mixture (per ml.): R-5-P or 6-PG, 10 μM.; glycylglycine buffer pH 7.85, 25 μM.; $MgCl_2$, 5 μM.; DPN or TPN, 10 μM.; extract, 0.3 ml. (12 mg. protein). Total volume, 10 ml; samples taken, 1 ml.; protein precipitation with 0.5 ml. 20% TCA. Chromatography, elution, and analysis as described under *Analytical Methods.*

of D-ribulose was obtained and no evidence for free D-xylulose or free D-ribose could be found. Perhaps Ru-5-P acts as a reservoir of ketopentose phosphate under these conditions.

The disappearance of SH-7-P once formed is in keeping with the further utilization of this compound in the transaldolase and other subsequent reactions.

It should be noted, however, that when 6-PG was used as the substrate in the presence of added TPN only about 26% of the theoretically possible heptulose was formed (Fig. 4). This suggests that the pentose cycle, although it operates in these extracts, does not account for a great deal of 6-PG utilization. When sodium fluoride was added to the 6-PG+TPN system, thus eliminating the possible utilization of 6-PG via the Entner–Doudoroff pathway (23, 24, 25), a little extra SH-7-P is formed. It appears that entry of 6-PG into the pentose cycle is probably controlled by two factors: (*a*) the extent to which 6-PG enters the Entner–Doudoroff pathway and (*b*) the actual quantity of 6-PG dehydrogenase present. The latter is likely to become the rate-limiting reaction in this system.

Hexosephosphate Formation from Ribose-5-phosphate

It has been shown previously that the transketolase and transaldolase reactions will take place anaerobically as well as they do aerobically (15).

Upon anaerobic incubation of 10 μM. R-5-P with *X. phaseoli* extract for 2 hours, 4.5 μM. of hexosephosphate and 4.2 μM. of triosephosphate are formed. Paper chromatograms suggest that the hexosephosphate fraction is a mixture of G-6-P and F-6-P, with a greater proportion in the form of F-6-P. The final phases of the pentose cycle are, therefore, operating satisfactorily under the conditions described.

Pyruvate Formation (Entner–Doudoroff) and Utilization

Katznelson (3) has already shown that pyruvate is formed readily from 6-PG in *X. phaseoli* extracts. In the present study additional substrates have been tested and factors involved in the utilization of pyruvate investigated. By far the fastest rate of pyruvate formation was obtained with 6-PG (Fig. 5), reaching a maximum at 30 minutes' incubation time. Other substrates (G-6-P, FDP, R-5-P, and PGA) resulted in much slower rates requiring much longer time periods to reach equilibrium conditions. As the data show, the addition of fluoride completely inhibits the formation of pyruvate. This would be the case whether pyruvate arises via the Entner–Doudoroff splitting system (23, 24) or via a portion of the glycolytic route. It will be noted that 10 μM. of 6-PG or G-6-P give rise to a theoretical 10 μM. of pyruvate when

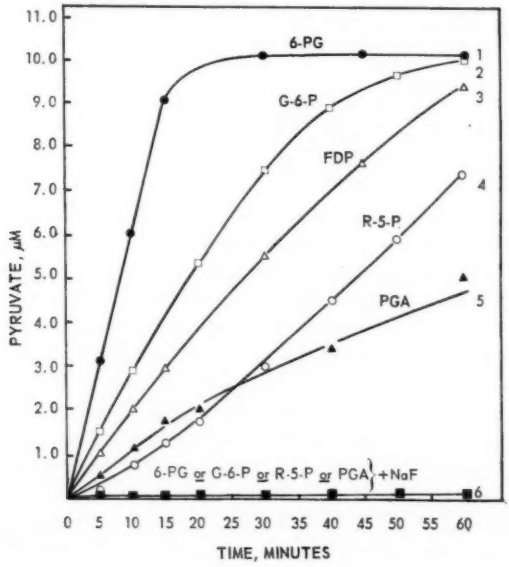

FIG. 5. Formation of pyruvate during incubation of various substrates with frozen-cell extracts.

Components of initial reaction mixture (per ml.): substrates, 10 μM.; glycylglycine buffer pH 7.8, 20 μM.; $MgCl_2$, 5 μM.; all cofactors, 5 μM. each; NaF (where used), 50 μM.; extract of previously frozen cells, 0.3 ml. (12 mg. protein). Total volume, 10 ml.; samples taken, 1 ml.; protein precipitation with 2 ml. 10% TCA. Cofactors used as follows: 1 = none; 2, 3 = TPN; 4 = TPN + DPN + ATP; 5 = TPN + DPN + ADP; 6 = all substrates as indicated, plus NaF.

TABLE II

DISAPPEARANCE OF PYRUVATE DURING 90 MINUTES INCUBATION AT 30° C. (pH 7.2) WITH EXTRACTS OF *Xanthomonas phaseoli* (INITIAL PYRUVATE CONCENTRATION = 10 μM.)

Conditions	Net disappearance, μM.
A. Frozen cell extracts	
No cofactors added to the extract	0.16
All cofactors added to the extract (DPN, TPN, ATP, lipoate, ThPP, CoA)	0.22
B. Extracts of fresh cells	
No cofactors added to the extract	0.92
All cofactors added to the extract (DPN, TPN, ATP, lipoate, ThPP, CoA)	9.70
All cofactors except DPN	9.87
All cofactors except TPN	9.95
All cofactors except ATP	9.04
All cofactors except lipoate	9.75
All cofactors except ThPP	6.58
All cofactors except CoA	6.00
All cofactors except ThPP + CoA	0.68

NOTE: Components of initial reaction mixture (per ml.): phosphate buffer pH 7.2, 20 μM.; $MgCl_2$, 5 μM.; DPN, TPN, ATP, CoA, lipoic acid, and ThPP, 0.3 μM.; extract of fresh cells, 0.2 ml. (8 mg. protein). Total volume, 10 ml.; samples taken, 1 ml.; protein precipitation with 2 ml. 10% TCA. Pyruvate (10 μM.) added at zero time.

DPN is not added. In the presence of added DPN (5 μM.), however, these substrates give rise to 14.9 and 15.5 μM. pyruvate respectively. This suggests that approximately one-half of the triosephosphate which arises as a result of the splitting reaction is also converted to pyruvate when DPN is added. This amount approximates closely the amount of PGA conversion to pyruvate (Fig. 5, curve 5). Arsenite added in any of these experiments had no effect at all. This is understandable since the above results show (curve 1) that in frozen-cell extracts pyruvate, once formed, does not disappear again under these conditions. Although this was a fortunate situation which enabled us to obtain the above results, it was also of interest to discover under what conditions pyruvate would be oxidized further. In Table II are summarized the results of experiments with fresh and frozen cell preparations and the effects of specific cofactors on the pyruvic oxidase system. It is readily seen that addition of DPN, TPN, ATP, lipoate, ThPP, and CoA did not restore activity in the frozen-cell extract. In the fresh-cell extract, however, almost complete pyruvate oxidation occurred when these cofactors were added together. Moreover, the effect has been traced to the specific requirement for added ThPP and CoA.

The Question of Classical Glycolysis

(*a*) *Phosphoglucose Isomerase*

In view of the presence of a highly active G-6-P dehydrogenase in our extracts, it was possible to obtain reliable estimates of phosphoglucose isomerase

by use of the spectrophotometric method (Table III). Thus, F-6-P in the presence of the isomerase is converted to G-6-P, which, in turn, acts as the substrate in the dehydrogenase assay with TPN. Thus, the comparative effects of TPN and DPN as cofactors as well as the DPN+cyanide systems, seen earlier (Fig. 1) are reproduced here but with F-6-P as the initial substrate. The presence of phosphoglucose isomerase is, therefore, established although by indirect means.

TABLE III

EVIDENCE FOR THE PRESENCE OF PHOSPHOGLUCOSE ISOMERASE IN *X. phaseoli* EXTRACTS, USING THE SPECTROPHOTOMETRIC TPN-REDUCTION METHOD

Conditions	Net optical density at completion of reaction (3 min.)
F-6-P + TPN	0.570
F-6-P + DPN	0.121
F-6-P + DPN + cyanide	0.456

NOTE: Components (in 3 ml.): glycylglycine buffer pH 7.85, 100 μM.; $MgCl_2$ 10 μM.; F-6-P, 10 μM.; sodium cyanide, 150 μM.; pyridine nucleotides, 0.3 μM.; 0.05 ml. fresh enzyme preparation (containing 6 mg. protein and 5 μM. GSH).

(*b*) *Phosphofructokinase and Aldolase*

Phosphofructokinase was investigated by two independent methods. The manometric method of Colowick and Kalckar (28) showed that only about 12.5% of the theoretical gas evolution took place (Table IV) in a system in which excess substrate and enzyme were used, but in which the phosphate donor (ATP) was the limiting factor.

TABLE IV

MANOMETRIC DETERMINATION OF PHOSPHOFRUCTOKINASE ACTIVITY IN *X. phaseoli* EXTRACTS

Substrate	Concn., μM.	Phosphate donor	Concn., μM.	Theoretical CO_2 evolution, μl.	Actual net CO_2 evolution, μl.	% of theory
F-6-P	25.0	ATP	14.4	324	42	12.9
F-6-P	25.0	ATP	7.2	162	20	12.3

NOTE: Additional components (in 3 ml.): bicarbonate buffer, 60 μM.; $MgCl_2$, 10 μM.; sodium fluoride, 60 μM.; 1.0 ml. fresh enzyme preparation (containing 45 mg. protein and 10 μM. GSH). Atmosphere: 95% nitrogen+5% carbon dioxide.

An active aldolase (Table V, Expts. 2 and 4) was found in these extracts using the chromogen method of Sibley and Lehninger (17). For preparation of a standard curve, pure dihydroxyacetone was used since it has been shown (17) to be the chief chromogen-producing component in the triosephosphate – dihydroxyacetone phosphate mixture. For purposes of calculation of the

TABLE V

PHOSPHOFRUCTOKINASE AND ALDOLASE ACTIVITIES IN EXTRACTS OF *X. phaseoli* AFTER 20 MINUTES REACTION

Expt.	Type of extract used	Substrate	Concn. of substrate (as the Ba salt), μM.	Net Klett Units following reaction	Yield, as dihydroxy-acetone, μM.	Theoretical maximum yields, as free dihydroxyacetone, μM.	% Completion of reaction
1	E	F-6-P	12.65	46	1.25	10.50	9.80
2	E	FDP	12.65	237	6.45	7.12	90.60
3	BE	F-6-P	12.65	4	0.10	10.50	0.95
4	BE	FDP	12.65	2	0.05	7.12	0.70

NOTE: Components (per ml.): substrate concn. (as shown); tris buffer, pH 8.6, 20 μM.; $MgCl_2$, 6 μM.; hydrazine sulphate (neutr.), 28 μM.; ATP (Expts. 1 and 3 only), 10 μM.; extract, 0.15 ml. (6 mg. protein). Total volume, 5 ml.; samples taken, 1 ml.; protein precipitation, with 2 ml. 10% TCA. E = fresh extract, BE = boiled extract.

theoretically possible maximum yields the equilibrium conditions first established by Meyerhof (40) were taken into account. Table V shows that only 9.8% conversion took place from F-6-P to the chromogenic triosephosphate, a value which is in fairly good agreement with the results of the manometric experiments in which 12.5% conversion (Table IV) took place. The phosphofructokinase, although apparently present, is only capable of a very small utilization of F-6-P and appears to be the bottleneck to the classical glycolytic reaction sequence in these extracts.

(*c*) *Conversion of Phosphoglyceric Acid to Pyruvate*

These reactions as well as their fluoride sensitivity have already been shown to occur in this organism (Fig. 5).

(*d*) *Hexosediphosphate Metabolism*

When FDP was used as substrate in several spectrophotometric experiments it was found that both DPN and TPN were reduced. The DPN reduction was IOA-sensitive whereas the TPN-linked system was completely insensitive to this inhibitor (Table VI). These results suggested that when DPN is used, the GAP–dehydrogenase system is measured while the G-6-P dehydrogenase system is the one catalyzing FDP oxidation in the presence of TPN. Thus FDP seemed capable of being metabolized in two directions depending upon the cofactor present. Since the equilibrium for phosphofructokinase is strongly in favor of FDP formation and since phosphofructokinase activity was extremely low (Table V), it appeared that FDP utilization via "Zwischenferment" was the least likely to occur unless some way could be discovered by means of which G-6-P could be formed from FDP without involving reversal of the phosphofructokinase reaction. In the course of these studies we found that a high concentration of fluoride (0.3 *M*) could be used to inhibit the TPN-linked, IOA-insensitive system. This suggested the possibility that a phosphatase might be involved since none of the known

TABLE VI

PYRIDINE NUCLEOTIDE REDUCTION WITH FRUCTOSE DIPHOSPHATE AS SUBSTRATE

Additions	Net optical density after 10 min.
DPN	0.438
DPN + IOA	0.039
TPN	0.542
TPN + IOA	0.540

NOTE: Cuvette components (in 3 ml.): sodium pyrophosphate buffer pH 8.5, 50 μM.; cysteine, 40 μM.; sodium arsenate, 40 μM.; IOA (where added), 40 μM.; FDP, 10 μM.; sonic extract of frozen cells, 0.1 ml. (containing 6 mg. protein).; DPN or TPN, 0.3 μM.; (Note: 7 min. incubation time allowed prior to nucleotide addition).

enzymes between FDP and G-6-P are fluoride-sensitive. An alkaline phosphatase for FDP was subsequently discovered in *X. phaseoli* extracts which specifically removes the phosphate grouping in carbon-1 of FDP thus resulting in F-6-P, which, in the presence of the active phosphoglucose isomerase gives rise to G-6-P. The removal of inorganic phosphate from FDP by this enzyme is illustrated in Fig. 6. When the reaction was carried out at pH 9.0 (curve 1) the highest initial activity was obtained but at pH 7.85 (curve 2) only slightly lower activity occurred. The latter assumes some importance since many of

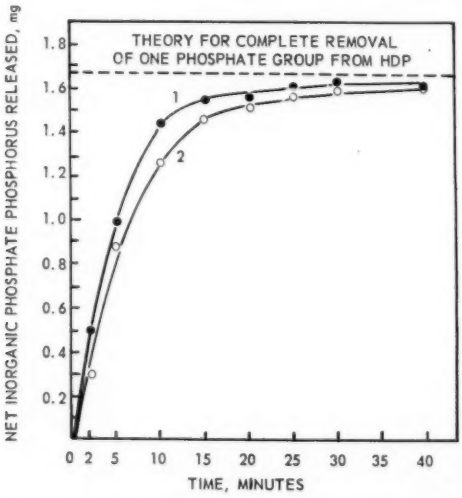

FIG. 6. The formation of inorganic orthophosphate from FDP by a phosphatase in *X. phaseoli* extracts.

Components of initial reaction mixture (in 9 ml.): buffer, 300 μM.; $MgCl_2$, 50 μM.; FDP, 53.8 μM.; fresh cell extract, 2.0 ml. (100 mg. protein). FDP and enzyme preparation (alone) served as blanks which were subtracted from the FDP+enzyme system. Samples taken, 1 ml.; protein precipitation with 1 ml. 10% TCA; 0.3 ml. aliquots used for analysis. Curve 1: tris buffer, pH 9.0; Curve 2: glycylglycine buffer, pH 7.85.

our experiments were done at or near pH 7.8. It should be noted, in addition, that when F-6-P was used as substrate, no free inorganic phosphate formation occurred.

Paper chromatographic studies were also carried out using the methyl cellosolve – pyridine – acetic acid – water (80:40:10:10) system recently described (8). R_f values so obtained were 0.12, 0.16, 0.22, 0.29, and 0.87 for FDP, 6-PG, G-6-P, F-6-P, and pyruvate respectively. The initial spot of R_f 0.12 gave rise to spots of R_f 0.16, 0.22, 0.29, and 0.87. After 20 minutes incubation, no FDP spot could be detected, suggesting that dephosphorylation in the C-1 position had been completed.

Contribution of Classical Glycolysis to Glucose-6-phosphate Oxidation

It is difficult to obtain a quantitative assessment of the contribution made by glycolysis to G-6-P utilization, since all inhibitors so far known for glycolysis will also cause a similar inhibition in the Entner–Doudoroff system. Also, since the triosephosphate moieties formed by either pathway would be expected to bear the same labelling if radioactive G-6-P were used, this approach offers little promise.

The only indirect evidence which we have so far shows that pyruvate formation from G-6-P in the presence of added DPN leads to 17% less pyruvate than the control, when IOA is added. But the conversion of 6-PG to pyruvate is inhibited by the same concentration of IOA to the extent of only 12%. It is possible, although by no means proved, that the difference of 5% could represent the extent to which glycolysis functions under these conditions.

Aerobic Oxidation of Some Key Metabolic Intermediates

The results in Table VII show that all the hexosephosphates used here are oxidized at approximately the same rate (during the first 2 hours). Further-

TABLE VII

AEROBIC OXIDATION OF SUBSTRATES BY EXTRACTS OF *X. phaseoli*

Substrates, 5 μM.	Net O_2 uptake at 120 min., μl.
G-6-P	223
F-6-P	198
FDP	235
6-PG	190
R-5-P	147
PGA	139
Pyruvate	155
Acetate	9
Citrate	267
α-Ketoglutarate	275
Succinate	275
Malate	215

NOTE: Components (in 3 ml.): phosphate buffer, pH 7.2, 100 μM.; $MgCl_2$, 20 μM.; ATP, 10 μM.; ADP, 2 μM.; DPN, 3 μM.; CoA, 1 μM.; ThPP, 4 μM.; lipoic acid, 4 μM.; fresh cell extract, 1.0 ml. (containing 42 mg. protein and 10 μM. GSH).

more, intermediates of the tricarboxylic acid cycle are also oxidized well, suggesting that the cycle is available for oxidative purposes. It may be pointed out that the extract used here was obtained from glucose-grown cells which did not oxidize acetate. There is a considerable difference in the extent to which FDP is oxidized in our extracts as compared to similar extracts of *Pseudomonas fluorescens* (31) or of *Acetobacter melanogenum* (41) in which relatively little oxidation took place. Further work on the individual enzymes of the tricarboxylic acid cycle and associated reactions is now in progress in our laboratory.

Discussion

From the evidence presented it appears that in cell-free extracts of *X. phaseoli* G-6-P is chiefly oxidized via "Zwischenferment" followed by the Entner–Doudoroff (23) splitting system to triosephosphate and pyruvate, which are then dissimilated further, and from the oxidation of which energy is derived. Some hexosephosphate is broken down by way of the pentose cycle, thus making available other sugar phosphates which may be required for vital processes. The classical glycolytic route does not appear to be of any real significance under the conditions studied. The tricarboxylic acid cycle appears to be operative.

So far, it has not yet been possible to find an active pyridine nucleotide transhydrogenase (24) in these extracts. This is a most interesting finding especially since this enzyme was first discovered in *Pseudomonas fluorescens*, an organism which displays many characteristics similar to those of *X. phaseoli*. Our results also show that DPNH oxidase activity is always very great, though some variations from one experiment to the next usually appeared, whereas TPNH oxidation proceeded at only about one-tenth the rate of the oxidation of DPNH. In the absence of a transhydrogenase, therefore, a most interesting question arises as to the mechanism of the terminal oxidation of DPNH and of TPNH. The inability of DPNH to reduce TTC in the presence of cell-free extracts also assumes some importance, especially since TTC was reduced to the red formazan by extracts of *P. fluorescens* (31). This suggests, though it does not prove, that DPNH oxidation may not be mediated by the usual type of flavoprotein.

It has recently been stated (42) that oxidative phosphorylation in liver mitochondria is associated with DPNH but not with TPNH oxidation. It was suggested that one of the key roles of the transhydrogenase was to transform TPNH into DPNH in order that oxidative phosphorylation may then take place. Recently, however, there have appeared several papers (43, 44, 45) suggesting strongly that TPNH oxidation is, indeed, associated with oxidative phosphorylation in beef heart, rat liver, and thoracic mitochondria of the bee. On the basis of the results presented in this paper it would appear that *X. phaseoli* should be a useful test organism for the study of oxidative phosphorylation unhampered by pyridine nucleotide transhydrogenase.

At first glance, the high concentrations of cyanide required to inhibit DPNH oxidase completely appear to be somewhat unusual. However, work of Slater

(46) with a heart muscle preparation shows a fairly high requirement for complete inhibition, extracts of *E. coli* showed no inhibition at 2×10^{-3} *M* KCN (47), and recent work of Vernon and White (48) indicates that the DPNH oxidase of *Micrococcus denitrificans* is completely cyanide-insensitive.

The specific FDP phosphatase described in this paper appears somewhat different from that found in extracts of *Acetobacter suboxydans* (49). The *Xanthomonas* enzyme shows a pH optimum near 9.0 but very little drop in activity at or near 7.8, whereas the *Acetobacter* enzyme is almost totally inactive at pH 7.8.

Although it has not been discussed in detail, hexokinase appears to be present in extracts of *X. phaseoli*. Rapid TPN reduction ensues spectrophotometrically when glucose, ATP, Mg^{++}, and the enzyme preparation are placed together into a cuvette at pH 7.8. In the presence of excess glucose and ATP, theoretical TPNH formation takes place in 2 minutes with 2 mg. protein.

With the information presented in this paper as background material, it is tempting to speculate on the question of the natural regulation and energetics of existing pathways of G-6-P oxidation. In view of the findings that the pentose cycle plays only a minor role in extracts of this organism, that phosphohexokinase activity is very low, that the organism contains a very active, specific phosphatase for FDP, and that the Entner–Doudoroff pathway is extremely active, it appears possible that a new cyclic process occurs here. The functioning of the proposed "hexose cycle" is given in Fig. 7. It is based on the following considerations:

(*a*) Reactions 1 and 2, leading to the formation of 6-PG, proceed readily to completion under our experimental conditions in presence of TPN (Fig. 1A). Glaser and Brown (50) determined the equilibrium constant of the reaction, G-6-P + TPN = TPNH + H^+ + 6-phosphoglucono-δ-lactone at pH 6.4 and at 28° C. However, at pH 7.8 (at which our experiments were conducted) the lactone will be still more stable and the reaction will tend to go still further to the right. Brodie and Lipmann (51), in studies of their purified gluconolactonase, have indicated that 100% lactone hydrolysis occurs in 5 minutes.

(*b*) The equilibrium of the formation of 2-keto-3-deoxy-6-phosphogluconate (KDPG) from 6-PG by 6-PG dehydrase (reaction 3) has been found by Kovachevich and Wood (24) to be completely toward formation of KDPG.

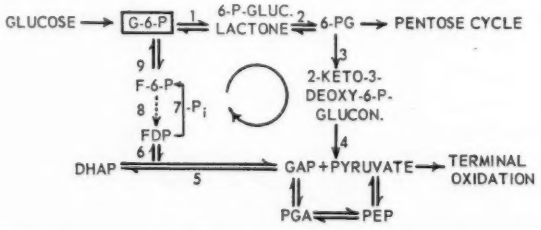

FIG. 7. Cyclic regeneration of hexosephosphate from triosephosphate as a major metabolic pathway in *X. phaseoli*.

(*c*) The same authors (25) have shown that at pH 7.6 in tris buffer the KDPG–aldolase reaction (reaction 4) also goes readily to completion, favoring strongly GAP and pyruvate formation.

(*d*) The equilibrium of triosephosphate isomerase (reaction 5) was shown by Meyerhof and Junowicz-Kocholaty (52) to favor strongly the formation of DHAP ($K_{\text{isomerase}}=[\text{DHAP}]/[\text{GAP}]=34.5$). The same authors published evidence for $K_{\text{aldolase}}=[\text{GAP}]\,[\text{DHAP}]/[\text{FDP}]=0.8\times10^{-4}$ at 30° C. and at pH 7.3. Therefore, the aldolase reaction (reaction 6), at equilibrium, favors strongly the formation of FDP. Although the equilibrium was found to be extremely temperature-dependent, at 20° C. and at a total phosphate ester concentration of 4.25 moles, only 32.5% of the equilibrium system was found to exist in the form of the triose ester. These equilibria, then, favor FDP formation rather than its utilization.

(*e*) Since it has been shown by us that phosphofructokinase activity (reaction 8) is very low in extracts of *X. phaseoli* but that FDP phosphatase is very active, and since phosphatase reactions in general are known to favor the dephosphorylated product, it follows that F-6-P formation from FDP (reaction 7) is a reaction which is thermodynamically likely to occur.

(*f*) Lohmann's (53) equilibrium established for phosphoglucose isomerase (reaction 9) as 70/30 in favor of G-6-P provides further evidence for the likelihood of such a system operating, as it were, "in reverse of glycolysis". Thermodynamically, the reactions between G-6-P and GAP are more likely to occur in reverse than in the forward direction. Krebs (54) has noted that most metabolic cyclic processes include at least one step which, for all practical purposes, is irreversible. He suggested that it is this step which controls the direction of the cycle even though constituent reactions may occur in the opposite direction. In our case, the FDP–phosphatase is one of the reactions which, for all practical purposes, is irreversible and therefore assigns the major direction to the cycle. Furthermore, 6-PG dehydrase and KDPG aldolase have similar tendencies, further reinforcing the view expressed in this paper. When taken together, the above reasons and the experimental results are taken to suggest the occurrence of a cyclic regeneration of hexosephosphate from triosephosphate via the Entner–Doudoroff splitting system in extracts of *X. phaseoli*.

The proposed cycle is considered to be an alternative, in cells in which the pentose cycle is not of great significance, to the hexose resynthesis from GAP proposed by Racker (55) to go via the pentose cycle. In *X. phaseoli* the cycle provides pyruvate which is then further metabolized by means of the TCA cycle and GAP, which is resynthesized into G-6-P. The latter is then oxidized, probably by TPN, which, if found to be linked to oxidative phosphorylation, would lead to ATP formation and thus greatly increase the working efficiency of the proposed cycle.

Great difficulties have been encountered in the past in attempts to interpret results of metabolic experiments with crude extracts carried out with C^{14}-labeled glucose (55, 56). Divergencies of opinion rather than of fact (57, 58)

have developed and it is considered possible that the extensive recycling which has often helped to make interpretation difficult may have been due to recycling of the triose- and hexose-phosphate moieties as described here. It is also of interest to recall that liver tissue contains a similar, very active, FDP phosphatase (59, 60).

Acknowledgment

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References

1. KATZNELSON, H. J. Bacteriol. **70**, 469 (1955).
2. KATZNELSON, H. J. Bacteriol. In press.
3. KATZNELSON, H. Nature, **175**, 551 (1955).
4. KRAGHT, A. J. and STARR, M. P. J. Bacteriol. **64**, 259 (1952).
5. STARR, M. P., DELEY, J., and KILGORE, W. W. Science, **125**, 929 (1957).
6. ZAJIC, J. E., DELEY, J., and STARR, M. P. Bacteriol. Proc. (Soc. Am. Bacteriologists), 116 (1956).
7. SEEGMILLER, J. E. and HORECKER, B. L. J. Biol. Chem. **192**, 175 (1951).
8. RUNECKLES, V. C. and KROTKOV, G. Arch. Biochem. Biophys. **70**, 442 (1957).
9. BALLOU, C. E. and FISCHER, H. O. L. J. Am. Chem. Soc. **77**, 3329 (1955).
10. ZILL, L. P. and TOLBERT, N. E. J. Am. Chem. Soc. **76**, 2929 (1954).
11. NEISH, A. C. Personal communication.
12. KLEVSTRAND, R. and NORDAL, A. Acta. Chem. Scand. **4**, 1320 (1950).
13. HOCHSTER, R. M. Can. J. Microbiol. **1**, 346 (1955).
14. DISCHE, Z. J. Biol. Chem. **204**, 983 (1953).
15. HOCHSTER, R. M. and STONE, B. A. Can. J. Microbiol. **2**, 132 (1956).
16. FRIEDEMANN, T. E. and HAUGEN, G. E. J. Biol. Chem. **147**, 415 (1943).
17. SIBLEY, J. A. and LEHNINGER, A. L. J. Biol. Chem. **177**, 859 (1949).
18. KING, E. J. Biochem. J. **26**, 292 (1932).
19. WARBURG, O. and CHRISTIAN, W. Biochem. Z. **303**, 40 (1939).
20. KORNBERG, A. and HORECKER, B. L. *In* Methods in enzymology. Vol. 1. *Edited by* S. P. Colowick and N. O. Kaplan. Academic Press, Inc., New York. 1955. p. 323.
21. HORECKER, B. L. and SMYRNIOTIS, P. Z. *In* Methods in enzymology. Vol. 1. *Edited by* S. P. Colowick and N. O. Kaplan. Academic Press Inc., New York. 1955. p. 326.
22. KREBS, E. G. *In* Methods in enzymology. Vol. 1. *Edited by* S. P. Colowick and N. O. Kaplan. Academic Press, Inc., New York. 1955. p. 407.
23. ENTNER, N. and DOUDOROFF, M. J. Biol. Chem. **196**, 853 (1952).
24. KOVACHEVICH, R. and WOOD, W. A. J. Biol. Chem. **213**, 745 (1955).
25. KOVACHEVICH, R. and WOOD, W. A. J. Biol. Chem. **213**, 757 (1955).
26. COLOWICK, S. P., KAPLAN, N. O., NEUFELD, E. F., and CIOTTI, M. M. J. Biol. Chem. **195**, 95 (1952).
27. KUN, E. and ABOOD, L. G. Science, **109**, 144 (1949).
28. COLOWICK, S. P. and KALCKAR, H. M. J. Biol. Chem. **148**, 117 (1943).
29. KATZNELSON, H. and SUTTON, M. D. J. Bacteriol. **61**, 689 (1951).
30. COHEN, S. S. *In* Chemical pathways of metabolism. Vol. 1. *Edited by* D. M. Greenberg. Academic Press, Inc., New York, N.Y. 1954. p. 173.
31. WOOD, W. A. and SCHWERDT, R. F. J. Biol. Chem. **206**, 625 (1954).
32. DEMOSS, R. D. and GIBBS, M. Bacteriol. Proc. (Soc. Am. Bacteriologists), 146 (1952).
33. DEMOSS, R. D., GUNSALUS, I. C., and BARD, R. C. J. Bacteriol. **66**, 10 (1953).
34. HOCHSTER, R. M. Arch. Biochem. Biophys. **66**, 499 (1957).
35. EICHEL, H. J. J. Biol. Chem. **222**, 121 (1956).
36. DELA HABA, G., LEDER, I. G., and RACKER, E. J. Biol. Chem. **214**, 409 (1955).
37. STONE, B. A. and HOCHSTER, R. M. Can. J. Microbiol. **2**, 623 (1956).
38. SRERE, P. A., COOPER, J. R., KLYBAS, V., and RACKER, E. Arch. Biochem. Biophys. **59**, 535 (1956).
39. HORECKER, B. L., HURWITZ, J., and SMYRNIOTIS, P. Z. J. Am. Chem. Soc. **78**, 692 (1956).
40. MEYERHOF, O. Bull. soc. chim. biol. **20**, 1033 (1938).
41. KATZNELSON, H. Can. J. Microbiol. **4**, 25 (1958).
42. KAPLAN, N. O., SWARTZ, M. N., FRECH, M. E., and CIOTTI, M. M. Proc. Natl. Acad. Sci. U.S. **42**, 481 (1956).

43. CHELDELIN, V. H., WIDMER, C., JOSHI, S., and NEWBURGH, R. W. J. Am. Chem. Soc. **79**, 1766 (1957).
44. VIGNAIS, P. V. and VIGNAIS, P. M. J. Biol. Chem. **229**, 265 (1957).
45. ERNSTER, L. and NAVAZIO, F. Biochim. et Biophys. Acta, **26**, 408 (1957).
46. SLATER, E. C. Biochem. J. **46**, 484 (1950).
47. PINCHOT, G. B. and RACKER, E. *In* Symposium on phosphorus metabolism. Vol. 1. *Edited by* W. D. McElroy and B. Glass. The Johns Hopkins Press, Baltimore, Md. 1951. p. 336.
48. VERNON, L. P. and WHITE, F. G. Biochim. et Biophys. Acta, **25**, 321 (1957).
49. HAUGE, J. G., KING, T. E., and CHELDELIN, V. H. J. Biol. Chem. **214**, 11 (1955).
50. GLASER, L. and BROWN, D. H. J. Biol. Chem. **216**, 67 (1955).
51. BRODIE, A. F. and LIPMANN, F. J. Biol. Chem. **212**, 677 (1955).
52. MEYERHOF, O. and JUNOWICZ-KOCHOLATY, R. J. Biol. Chem. **149**, 71 (1943).
53. LOHMANN, K. Biochem. Z. **262**, 137 (1933).
54. KREBS, H. A. Enzymologia, **12**, 88 (1946).
55. RACKER, E. Harvey Lectures, Ser. **51**, 143 (1957).
56. WOOD, H. G. Physiol. Revs. **35**, 841 (1955).
57. BLOOM, B., STETTEN, M. R., and STETTEN, D., JR. J. Biol. Chem. **204**, 681 (1953).
58. KATZ, S., ABRAHAM, R. H., and CHAIKOFF, I. L. J. Am. Chem. Soc. **76**, 2277 (1954).
59. GOMORI, G. J. Biol. Chem. **148**, 139 (1943).
60. POGELL, B. M. and MCGILVERY, R. W. J. Biol. Chem. **208**, 149 (1954).

CHEMICAL THERMOGENESIS IN THE FUNCTIONALLY EVISCERATED COLD-ACCLIMATED RAT[1]

FLORENT DEPOCAS

Abstract

The effect of functional evisceration (ligation of rectal colon, superior mesenteric and coeliac arteries, and portal vein) on the rise in oxygen consumption by rats which have been acclimated at 30° and at 6° C. and then anesthetized or curarized and exposed to 6° C. has been examined. At 30° C., the O_2 consumption of anesthetized cold-acclimated rats was higher than that of anesthetized warm-acclimated rats, but after functional evisceration there was no significant difference between the lower levels of O_2 consumption obtained in the two groups. In anesthetized warm-acclimated rats the increase in O_2 consumption in the cold after evisceration was smaller than that seen after sham-operation but both increases were suppressed by injection of tubocurarine. Anesthetized cold-acclimated rats, whether eviscerated or sham-operated, gave essentially the same increase in O_2 consumption when exposed to 6° C. and injection of tubocurarine did not lower the O_2 consumption to the levels observed before the cold-induced rise took place. In cold-acclimated curarized rats, functional evisceration before exposure to cold did not always prevent increases in O_2 consumption but the average increase was not significant in the group. Also, functional evisceration after increase in O_2 consumption had taken place in cold-acclimated curarized rats exposed to 6° C. did not immediately lower the O_2 consumption to the level observed after evisceration at 30° C. It is concluded that the presence of the abdominal viscera is not essential for the manifestation of chemical thermogenesis in the cold-acclimated white rat.

Introduction

It has previously been shown in this laboratory that warm- and cold-acclimated rats, anesthetized with barbital, could increase their oxygen consumption when transferred from a warm to a cold environment (7). Cold-acclimated rats doubled their O_2 consumption with little shivering while warm-acclimated rats showed an increase of only 50% while shivering violently. This evidence, taken in conjunction with that of Sellers *et al.* (14) on the decreased electrical activity of muscles of cold-acclimated rats in the cold implied a well-developed chemical thermogenesis, that is an increased heat production independent of muscular tone and shivering in such animals. This was further demonstrated by Cottle and Carlson (1), who showed that curarized cold-acclimated rats could maintain thermal balance at 5° C. while similar warm-acclimated rats could not raise their heat production to the same extent and quickly became hypothermic. The presence of a purely chemical thermogenesis in the cold-acclimated white rat is thus well substantiated. It remains to identify the tissue or tissues involved in this process.

In this study, the effect of functional evisceration on the rise in O_2 consumption elicited by cold exposure in anesthetized and curarized warm- and cold-acclimated rats has been examined. The rectal colon, the coeliac trunk, the superior mesenteric artery, and the portal vein were ligated in order to

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prevent blood flow through the stomach, intestines, spleen, pancreas, and liver. The relative ineffectiveness of this type of evisceration in completely preventing blood flow through the liver has been discussed by Peterson (12). However, more recent and quantitative evidence has been obtained by Russell (13), who showed that the liver was effectively cut off from the blood stream in this preparation.

Methods

Barbital-Anesthetized Rats

Thirty-one male Sprague–Dawley rats were randomly assigned to two lots, one maintained at 30° C. (16 rats, groups 1–3) and the other at 6° C. (15 rats, groups 4–6) for 3 to 6 weeks. The rats were kept in individual cages and Master Fox Chow and tap water were supplied ad libitum. After acclimation, when the warm-acclimated rats weighed on the average 341 g. (range 294–406 g.) and the cold-acclimated 294 g. (range 263–321 g.), anesthesia was induced with barbital and the rats were treated as described previously (7). One hour after induction of anesthesia, the rats were taped to a board in the supine position and the trachea was cannulated. Oxygen consumption was measured with a Pauling O_2 analyzer and an American Meter Co. dry test-meter. The P_{O_2} and volume of CO_2-free outlet gas were recorded, and the appropriate equation corresponding to these conditions (2) was used to calculate the O_2 consumed per unit time.

After 10 minutes the abdominal cavity was opened and threads were passed under the rectal colon, the coeliac and superior mesenteric arteries, and the portal vein. The abdominal incision was then simply covered with absorbent cotton. Thirty-five minutes after the start, the animals were either: (1) eviscerated by tightening the threads already surrounding the blood vessels and left in the 30° C. room (groups 1 and 4) or (2) eviscerated and transferred to a room at 6° C. (groups 2 and 5) or (3) simply transferred to the 6° C. room (groups 3 and 6) and O_2 consumption measurements continued for an additional 55 minutes. Shivering was also measured on the cold-exposed rats with safety-pin electrodes as described previously (7).

Curarized Rats

Treatment of the 18 cold-acclimated rats (average weight after 3 to 6 weeks acclimation: 276 g., range 243–331 g.) used in this series was essentially as described by Cottle and Carlson (1) except that all O_2 consumption values were obtained from measurements on the CO_2-free exhaled gases. A miniature Starling Ideal respirator with added check valve on the inspiration side was used at a tidal volume of 2.8 ml. on the average. Breathing rate was varied so as to maintain a P_{O_2} of 120–130 mm. Hg in the exhaled CO_2-free gases. Tubocurarine chloride, 360 μg. per rat (300 μg. per ml.) was injected very slowly into the femoral vein initially and half this dose was again injected at the first signs of visually observed activity. The cold-exposed animals were kept in a box designed so that when transferred to a cold room at 6° C. the air temperature within the enclosure dropped from 30° C. to 6° or 7° C. in

about 40 minutes. The evisceration technique used on the barbital-anesthetized rats was also used in this series, the blood vessels being prepared for ligation 45 minutes after initial curarization. Treatment of the animals was as follows: group 1, eviscerated at 94 minutes and left at 30° C.; group 2, eviscerated at 94 minutes and transferred to the cold room; group 3, transferred to the cold room at 94 minutes and eviscerated at 183 minutes. Rectal temperature was measured continuously in all rats with a copper–constantan thermocouple inserted to a depth of 6 cm.

Barbital-Anesthetized Rats Curarized in the Cold

Anesthesia of the 14 30° and 6° C. acclimated rats (respective average weights after 5 to 7 weeks acclimation: 359 g., range 345–394 g. and 302 g., range 272–343 g.) was induced with barbital as described previously (7). Ten minutes after tracheotomy all the animals were prepared for evisceration and all groups were transferred to a cold room at 6° C. 46 minutes later. The blood vessels were ligated in groups 1 and 3 before cooling but were left intact in groups 2 and 4. Sixty minutes after transfer to the cold room all the animals were curarized by slow intravenous injection of 360 μg. of D-tubocurarine chloride. Oxygen consumption was measured throughout the experiment as described above for "barbital-anesthetized rats" and for "curarized rats" under the two conditions. Rectal temperature was obtained in the usual way. Shivering was also measured with safety-pin electrodes as described previously (7) during exposure to 6° C., until curarization.

Results

Barbital-Anesthetized Rats

The O_2 consumption and the muscle electrical activity of the different groups of rats are given in Fig. 1. At 30° C., the average O_2 consumption of all the 6° C. acclimated rats (4.88 ml. O_2 per minute) exceeded that of all the 30° C. acclimated rats (4.27 ml. O_2 per minute), the difference 0.61 ± 0.15 achieving the 1% level of statistical significance. When the sham-operated rats (groups 3 and 6) were transferred from 30° to 6° C., the increases in O_2 consumption (averaged between the 56th and 84th minute of observation) were respectively 4.08 ± 0.45 and 7.25 ± 0.66 ml. O_2 per minute in the two acclimation groups while shivering was much more intense in the warm-acclimated rats, a behavior noted in the previous study (7). Evisceration at 30° C. (groups 1 and 4) produced a larger drop in O_2 consumption in 6° C. acclimated rats (2.34 ± 0.19 ml. O_2 per minute) than it did in the 30° C. acclimated rats (1.64 ± 0.26 ml. O_2 per minute) but the level attained by the two groups after evisceration was not significantly different. Exposing the eviscerated animals to an environment at 6° C. still elicited increases in O_2 consumption (groups 2 and 5). The increase above the level of the eviscerated rats left at 30° C. was 1.86 ± 0.59 for the 30° C. acclimated rats and 7.33 ± 0.35 ml. O_2 per minute for the 6° C. acclimated rats (values calculated from average O_2 consumption between 56th and 84th minute of exposure). There was no significant difference between

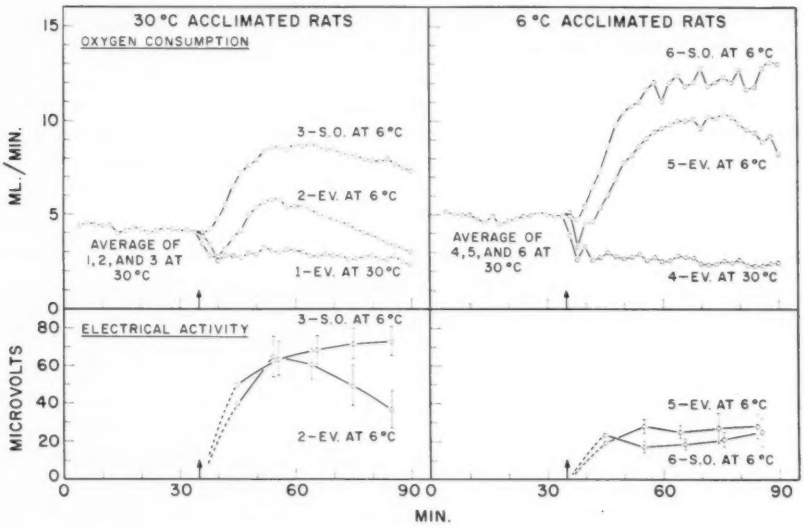

FIG. 1. Average O_2 consumption and muscle electrical activity of barbital-anesthetized 30° and 6° C. acclimated rats at 30° C. (average O_2 consumption of all similarly acclimated rats given for interval 0–35 min.), at 30° C. after evisceration (groups 1 and 4), at 6° C. after evisceration (groups 2 and 5), and at 6° C. after sham-operation (groups 3 and 6). Arrow indicates time of evisceration or transfer to 6° C. or both. All groups consisted of five animals except group 3 in which there were six.

the increases in O_2 consumption given by the eviscerated and the sham-operated cold-acclimated rats exposed to cold (respectively 7.33 ± 0.35 and 7.25 ± 0.66 ml. O_2 per minute). Also there was no significant difference between the levels of shivering in the two groups of cold-acclimated rats throughout the exposure to 6° C. Both groups of warm-acclimated rats showed the same initial higher level of shivering, which was maintained in the sham-operated rats (group 3) but decreased significantly with time in the eviscerated ones (group 2).

Curarized Rats

The O_2 consumption and the rectal temperatures of the three groups of curarized cold-acclimated rats are given in Fig. 2. Sham-operated rats (group 3) almost doubled their O_2 consumption on cooling as rectal temperatures dropped from 37° to 35° C. Eviscerating the curarized cold acclimated rats at 30° C. (group 1) lowered the O_2 consumption by approximately 39%. When the eviscerated curarized rats were transferred to the cold environment (group 2) the response was variable; five rats increased their O_2 consumption (range of maximal increase: 0.6–5.1 ml. O_2 per minute) above the level attained after evisceration, and three showed a steady decline; the average for this group during cold exposure was above that of group 1 after evisceration at 30° C. but the difference between the two groups was not significant.

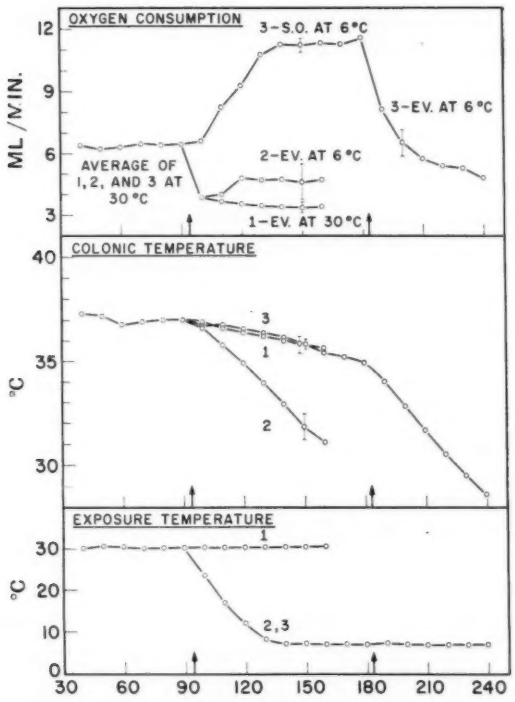

FIG. 2. Average O_2 consumption and rectal temperature of curarized 6° C. acclimated rats at 30° C. (average values of all rats given for interval 40–90 minutes), at 30° C. after evisceration (group 1, five rats), at 6° C. after evisceration (group 2, eight rats), and at 6° C. after sham-operation followed by evisceration (group 3, five rats). First arrow indicates time of evisceration or transfer to the cold-room or both. Second arrow indicates time of evisceration of animals in group 3. Average exposure temperature for the different groups of rats is also given.

Eviscerating the animals during exposure to the cold environment (group 3) produced a progressive fall in O_2 consumption but did not suppress abruptly all the increase induced by cold exposure. In group 3, rectal temperature also dropped precipitously at a rate of 1° C. per minute after evisceration.

Anesthetized Rats Curarized in the Cold

The average O_2 consumption, muscle electrical activity, and rectal temperature of the anesthetized rats, curarized after the increase in O_2 consumption due to cold exposure had taken place, are given in Fig. 3. The metabolic response pattern of the four groups of rats in this series, before curarization, was qualitatively similar to that obtained in the more extensive study of anesthetized rats reported above. The significant finding in this experiment is the effect of curarization on the cold-induced O_2 consumption. In the eviscerated (group 1) and the sham-operated (group 2) warm-acclimated rats, curarization precipitously lowered the O_2 consumption to levels below those

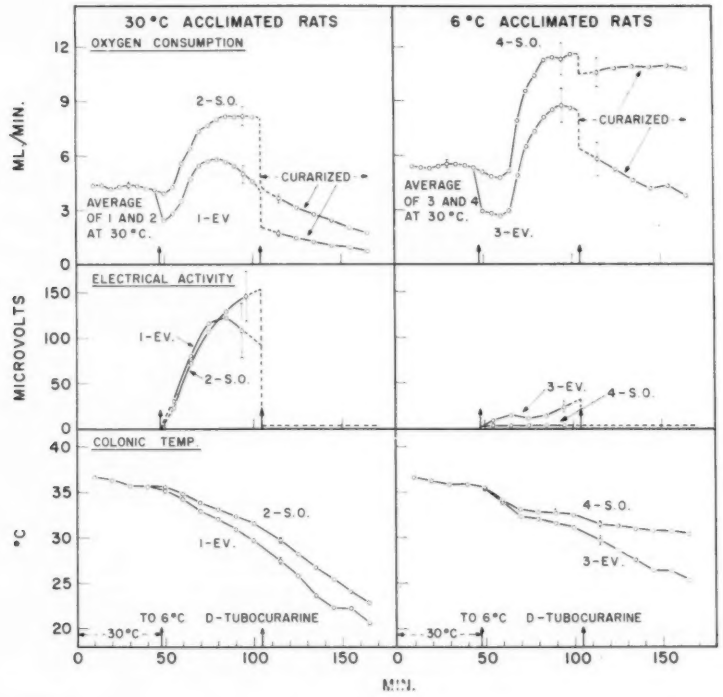

FIG. 3. Average O_2 consumption, muscle electrical activity, and rectal temperature of barbital-anesthetized 30° C. and 6° C. acclimated rats at 30° C. (average values of all similarly acclimated rats given for interval 10–45 minutes), at 6° C. after evisceration (groups 1 and 3), and at 6° C. after sham-operation (groups 2 and 4). First arrow indicates time of evisceration or transfer to the cold-room or both. Second arrow indicates time of curarization of all animals. There were 3 rats per group except group 3 in which there were five.

observed before the increase due to cold exposure while in the sham-operated cold-acclimated rats (group 4) it was lowered but little. In the functionally eviscerated cold-acclimated rats (group 3), O_2 consumption fell after curarization but stayed well above the value observed immediately after evisceration and transfer of the animal to the cold room. It is estimated roughly that curarization suppressed initially all the extra O_2 consumption induced by cold exposure in both groups of warm-acclimated rats. Similarly, curarization suppressed initially 42 and 16% of the maximal extra O_2 consumption induced respectively in the eviscerated and in the sham-operated cold-acclimated rats.

Discussion

Normal warm- and cold-acclimated rats raise their heat production very rapidly and maintain thermal balance for a few hours when exposed to cold environments at least down to −10° C., in which the heat production more than

doubles (3). In the warm-acclimated rat, which has hardly any chemical thermogenesis (1), this is probably accomplished mainly by shivering. The 6° C. acclimated rat still shivers after exposure for 5 weeks at −6° C. (6) but chemical thermogenesis alone seems adequate at 6° C. as the curarized cold-acclimated rat can almost maintain thermal balance at that temperature, as demonstrated by Cottle and Carlson (1), and as verified in the present work. However, the metabolic response to cold exposure is much slower in the curarized rat (and also in the anesthetized rat) than it is in the normal rat (3). In addition, the anesthetized rat has a lower metabolism at 30° C. than has the normal or curarized rat. Thermal balance is not maintained either when the anesthetized rat is transferred directly from a warm to a cold environment (7) or when cooled gradually (unpublished observations) as was done for curarized rats in this study. In spite of these defects, the anesthetized rat can still respond to cold exposure by a sharp increase in O_2 consumption.

In this study, the experiment on "barbital-anesthetized rats" shows clearly that anesthetized cold-acclimated rats, when functionally eviscerated, give a metabolic response of the same magnitude as that of sham-operated cold-acclimated rats on exposure to 6° C. (Fig. 1). It follows that in the anesthetized cold-acclimated rat, either the abdominal viscera (excluding the kidneys) are not involved in the O_2 consumption increase in the cold, or the remaining tissues compensate for their absence. In either case, the observation emphasizes the role of the non-visceral tissues as a possible site of chemical thermogenesis in the cold-acclimated rat. The possibility of a larger contribution of shivering to the metabolic response in the eviscerated than in the sham-operated 6° C. rats is unlikely as there was no significant difference in level of shivering of the two groups.

The ability of the functionally eviscerated rat to give a metabolic response to cold is even more astonishing when the profound metabolic disturbances resulting from evisceration are considered, especially as regards the function of the liver in providing other tissues with required substrates. This important function is well illustrated by the fact that the average survival time of eviscerated rats is only 136 minutes (13). This complicating factor could explain the decrease in O_2 consumption observed in the functionally eviscerated animals exposed to cold. However, the lack of maintenance of the O_2 consumption does not infirm the observation that the initial metabolic response is independent of the abdominal viscera in the anesthetized cold-acclimated rats.

As often observed previously for intact rats (5) and for curarized rats (1) at 30° C., the O_2 consumption of the anesthetized cold-acclimated rats was higher than that of warm-acclimated animals. The functionally eviscerated warm- and cold-acclimated rats, at 30° C., showed the same value of O_2 consumption, thus indicating that the viscera are responsible for the higher metabolic rate of the cold-acclimated rat at 30° C., a conclusion reached by You and Sellers (16) on the basis of O_2 consumption measurements on liver slices.

Results obtained with curarized cold-acclimated rats (Fig. 2) were not as clear-cut as with anesthetized rats. The cold-exposed functionally eviscerated rats had a higher average O_2 consumption than the eviscerated rats maintained at 30° C. but the difference between the two groups was not significant, thus indicating the absence of chemical thermogenesis in the eviscerated curarized cold-acclimated rat. Results obtained after evisceration of the cold-exposed sham-operated rats throw some doubt on this conclusion as the average O_2 consumption remained significantly above that of the eviscerated rats at 30° C. for at least 60 minutes after evisceration when the experiment was terminated. This last observation would indicate the persistence of some nonvisceral chemical thermogenesis. This set of results then appear to be internally inconsistent and preclude drawing definite conclusions. It has also been observed that curarization disturbs the carbohydrate metabolism of rats at least as manifested by the very high plasma glucose values found in this condition (unpublished experiments, and 9). The metabolic alterations due to evisceration added to those of curarization are possibly disturbing enough to lower the nonvisceral chemical thermogenesis response.

Results obtained with anesthetized warm-acclimated rats curarized after cold exposure, whether sham-operated or eviscerated (Fig. 3), are consistent with the hypothesis that shivering is the main process by which warm-acclimated rats increase their heat production in the cold. Also, the small drop in O_2 consumption observed after curarization of the cold-exposed sham-operated 6° C. rats (Fig. 3) is consistent with the hypothesis that chemical thermogenesis is the main process by which cold-acclimated rats increase their heat production in the cold. The O_2 consumption values observed after curarization of the functionally eviscerated anesthetized 6° C. rats are confusing. The slow decline in O_2 consumption noted after the initial drop of approximately 42% on curarization cannot be ascribed to the simple disappearance of shivering, which should be immediate, but again points to the disturbing effects of curarization associated with evisceration.

Definitive conclusions to be drawn from experiments involving both curare and evisceration in cold-acclimated rats are then dependent on further elucidation of the metabolic effects of curare which seem to occur in addition to the myoneural junction block. It can, however, be argued that the immediate lowering of O_2 consumption on curarization is the only effect directly related to disappearance of shivering. Accordingly, the presence of nonvisceral chemical thermogenesis can be considered demonstrated at least in the eviscerated cold-acclimated rats curarized after the rise in O_2 consumption had taken place (Fig. 3).

The above observations parallel those of Lyman and Chatfield (10), who demonstrated that curarized waking hibernators which are functionally eviscerated are still able to raise their cheek-pouch temperature to over 25° C. These authors consider the heart as a major heat-producing organ and also suggest the possibility that heat may be produced in muscle without physical activity. In the cold-acclimated white rat, in which the increase in heart

rate on exposure to cold is not as dramatic as that seen in the waking hibernator, the second possibility should be considered but it is difficult to devise experiments which would demonstrate this phenomenon directly.

The finding of a higher Q_{O_2} (15, 16) and succinoxidase activity of the liver (16) of cold-acclimated rats has been taken as evidence of the role of this organ in the increased capacity to produce heat by chemical thermogenesis seen in cold-adapted animals (8). These observations are probably more useful in explaining the higher metabolic rate of the cold-acclimated rat at 30° C., which could be expected to persist in excised tissues, than in relating them to the ability of the cold-acclimated rat to raise transiently its heat production by virtue of chemical thermogenesis alone. Emphasis of the role of the liver in chemical thermogenesis deduced from temperature measurements (4) is not very convincing as the same technique has been used to implicate the muscles (11). However, the possibility remains that many tissues are involved in chemical thermogenesis. The experiments reported here only show that it is possible, under certain conditions, to observe chemical thermogenesis in cold-acclimated rats deprived of their abdominal viscera.

Acknowledgment

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References

1. COTTLE, W. H. and CARLSON, L. D. Proc. Soc. Exptl. Biol. Med. **92**, 845 (1956).
2. DEPOCAS, F. and HART, J. S. J. Appl. Physiol. **10**, 388 (1957).
3. DEPOCAS, F., HART, J. S., and HEROUX, O. J. Appl. Physiol. **10**, 393 (1957).
4. FEDEROV, N. A. and SHUR, E. I. Am. J. Physiol. **137**, 30 (1942).
5. HART, J. S. Rev. can. biol. **16**, 133 (1957).
6. HART, J. S., HEROUX, O., and DEPOCAS, F. J. Appl. Physiol. **9**, 404 (1956).
7. HEROUX, O., HART, J. S., and DEPOCAS, F. J. Appl. Physiol. **9**, 399 (1956).
8. HSIEH, A. C. L. and CARLSON, L. D. Am. J. Physiol. **188**, 40 (1957).
9. HSIEH, A. C. L. and CARLSON, L. D. Am. J. Physiol. **190**, 243 (1957).
10. LYMAN, C. P. and CHATFIELD, P. O. J. Exptl. Zool. **114**, 491 (1950).
11. MAGNE, H. J. physiol. et pathol. gén. **16**, 360 (1914–15).
12. PETERSON, J. McI. Physiol. Revs. **14**, 586 (1934).
13. RUSSELL, J. A. Am. J. Physiol. **136**, 95 (1942).
14. SELLERS, E. A., SCOTT, J. W., and THOMAS, N. Am. J. Physiol. **177**, 372 (1954).
15. WEISS, A. K. Am. J. Physiol. **177**, 201 (1954).
16. YOU, R. W. and SELLERS, E. A. Endocrinology, **49**, 374 (1951).

THE DETERMINATION OF PROTEIN-BOUND IODINE[1]

G. A. ROBINSON AND H. A. DELUCA

Abstract

A method for the determination of protein-bound iodine in plasma is described. It is based essentially on the method of Barker, Humphrey, and Soley, which makes use of the catalytic effect of iodide on the reduction of ceric ion by arsenious acid. A number of improvements have been made in the direction of ease of manipulation and an over-all saving of time.

It is generally agreed that the level of protein-bound iodine in plasma is a good index of thyroid function. Its estimation, however, has not been universally adopted inasmuch as it is technically a difficult procedure. Most of the methods that have appeared recently in the literature are modifications of the procedure of Barker, Humphrey, and Soley (1). The present paper describes some changes in this procedure that not only have simplified the method but have considerably reduced the total time required for a determination. The method of Barker *et al.* (1) consists essentially of (*a*) a dry alkaline ashing of the precipitated plasma protein, (*b*) an acid leaching of the iodide from the remaining ash, and (*c*) colorimetric determination of the iodide by its catalytic effect on the reduction of ceric ion by arsenious acid.

Preparation of Reagents

Iodide standards are made up with doubly distilled water. Distilled water is used for all other solutions.

Zinc sulphate 10%.—100 g. $ZnSO_4.7H_2O$ per liter of solution.

Sodium hydroxide 2%.—20 g. NaOH per liter. These two solutions are adjusted so that 11.0 ± 0.2 ml. sodium hydroxide gives with 10.0 ml. zinc sulphate a faint pink color with phenolphthalein.

Sodium carbonate 20%.—200 g. Na_2CO_3 per liter.

Hydrochloric acid 4N.—400 ml. 37% C.P. reagent per liter of solution.

Sulphuric acid 2N.—56.0 ml. 96% C.P. reagent per liter.

Sodium arsenite 1%.—10 g. $NaAsO_2$ per liter.

*Ceric ammonium sulphate 2%.**—20 g. $Ce(NH_4)_4(SO_4)_4.4H_2O$ per liter. The 20 g. of ceric salt is dissolved in a solution consisting of 85 ml. of concentrated sulphuric acid in 500 ml. of distilled water. The solution is then brought up to a final volume of 1 liter.

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Contribution from the Department of Biochemistry, University of Western Ontario, London, Ontario, Canada.

*Obtainable from G. Frederick Smith Chemical Company, Columbus, Ohio.

Sodium iodide standards.—The sodium iodide is heated in an electric furnace at 300° C. for approximately two hours, then placed in a vacuum desiccator to cool. Solutions are prepared as follows:

(*a*) 100 μg. of iodine per ml. (118.1 mg. NaI per liter).

(*b*) 1.0 μg. of iodine per ml. (10 ml. of solution (*a*) per liter).

(*c*) 0.08 μg. iodine per ml. (8 ml. of solution (*b*) per 100 ml. of solution).

It is necessary that the weighing be done rapidly since the salt is deliquescent. Solutions (*a*) and (*b*) remain stable at refrigerator temperatures and (*c*) is prepared as required (approximately every two weeks).

Albumin solution 6.7%.—One gram of "egg albumin, Merck" is dissolved in 15 ml. of distilled water.

Method

(a) Test Material

Into a Pyrex culture tube (13×100 mm.) is pipetted 0.5 ml. of heparinized plasma.

(b) Precipitation and Washing

To the plasma sample is added 0.5 ml. of zinc sulphate solution. This mixture is shaken thoroughly; then 0.5 ml. of sodium hydroxide solution is pipetted into the tube, the contents of which are vigorously stirred with a glass rod. The sample is then washed by adding 5 ml. of distilled water. The tube is centrifuged for 5 minutes and the supernatant liquid is decanted and discarded. After four such washings, 0.5 ml. of 20% sodium carbonate is pipetted into each tube, which is stirred well. A few drops of distilled water are run down the rod to wash off any adhering precipitate.

(c) Drying

One of two alternative methods is used; the first requires less attention from the operator, whereas the other has the advantage of being more rapid.

Method A.—The sample is dried overnight in an oven at 70° to 90° C. This is the method described by Barker *et al.* (1).

Method B.—A simple quick-drying apparatus consisting of a hot plate and air jets (see later) was developed with which it is possible to dry samples sufficiently for ashing within ten or fifteen minutes.

(d) Ashing

Grinding the lump of dried protein with a heavy glass rod greatly increases the efficiency of the ashing procedure.

Ashing is done in an electric muffle furnace (Temco Model RCE) at 640 ± 10° C. for one and a half to two hours. In order to obtain uniform ashing, the tubes are supported off the floor of the furnace with a wire rack.

(e) Dissolving the Ash

To the cool ash, 0.5 ml. of 4 *N* hydrochloric acid is added slowly. When effervescence ceases, 2 ml. of 2 *N* sulphuric acid is pipetted in and mixed thoroughly. The tube is centrifuged for 5 minutes at approximately 2000 r.p.m. and the supernatant is decanted gently into a second tube.

(f) Colorimetry

The solutions are read in a colorimeter with a suitable filter. (A Fisher Electrophotometer Model A with micro-attachment using a 425 mμ filter was found satisfactory). Into each colorimeter tube is pipetted 0.5 ml. of 1% sodium arsenite solution, followed by 2 ml. of the acid supernatant containing the dissolved ash. The tubes are then warmed in a water bath at 39° C. for 15 minutes. A quantity of 2% ceric ammonium sulphate solution contained in a small bottle is also warmed.

At "zero" time, 1.0 ml. of the ceric solution is pipetted into the first colorimeter tube. The contents of the tube are mixed thoroughly by inversion, the tube being closed by a finger protected with a portion from a rubber glove. After the colorimeter tube is returned to the water bath, this finger is rinsed in distilled water and wiped dry. At time "0.5 minutes", the procedure is repeated with the second tube, and so on at 30-second intervals for a complete series of tubes. (A batch of 18 tubes were usually run at one time.) The optical density of each sample is read at exactly 10 minutes after mixing. The concentration of the protein-bound iodine of the plasma sample is obtained by applying the optical density reading to a curve of standards as described below.

(g) Preparation of Curve of Standards

In order to approximate the protein content of plasma, 0.5 ml. samples of 6.7% egg albumin are used in the following manner in preparing the curve of standards. The albumin is precipitated, then ashed, and the ash dissolved in the manner described for the plasma samples.

TABLE I

THE PREPARATION OF COLORIMETER TUBES FOR THE CURVE OF STANDARDS

	Tube Number					
	1 and 2	3 and 4	5 and 6	7 and 8	9 and 10	11 and 12
Ml. of 8 μg.% iodide standard	0	0.2	0.4	0.6	0.8	1.0
Iodide content of tubes, μg.	0	0.016	0.032	0.048	0.064	0.080
Ml. of water	1.0	0.8	0.6	0.4	0.2	0
PBI equivalent for graph, μg.%	0	4	8	12	16	20

A series of standard iodide solutions in colorimeter tubes are prepared as shown in Table I. These are dried in an oven at 90° C. To each tube is added 0.5 ml. of arsenite solution and 2.0 ml. of the solution from the ashed albumin samples. The remainder of the procedure is the same as that for treating the plasma samples at the same point. From the results obtained, a curve of optical density versus content of iodine is constructed (Fig. 1).

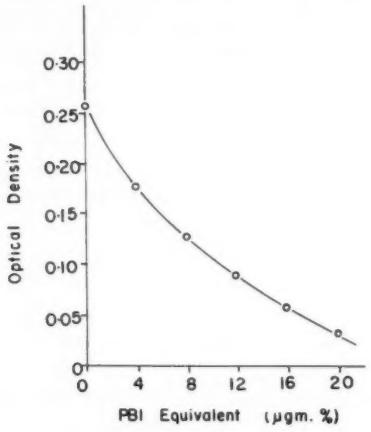

FIG. 1

Notes on Procedure

(a) Test Materials

Salter and McKay (2) reported that silica from the glass tubes inhibits the ceric–arsenious reaction. Barker *et al.* (1) used the tubes as many as four times before discarding them. In our hands, tubes used for a fourth time did not give satisfactory results. In view of this, new tubes were used for all determinations.

Serum or plasma from heparinized or oxalated blood is suitable for this estimation. Sodium citrate must be avoided as an anticoagulant since this substance interferes with the determination.

(b) Precipitation and Washing

Some of the sodium carbonate may come out of solution during cool weather so that it may be necessary to warm this reagent before using.

(c) Drying

The precipitated protein must be dried before it is ashed. The quick-drying apparatus, developed during this investigation, consists of 3-in. lengths of copper tubing (inside diameter 14 mm.) closely packed upright in a rectangular metal container. The latter rests on a small hot-plate with a variable temperature contol. (The copper tubing conducts heat around the sides of the test tubes for quick, even drying.) In order to suppress bubble formation, air jets directed vertically downwards are used. For a small number of tubes, jets with glass tips are convenient. For a large number of tubes a more elaborate arrangement may be desirable. For the present investigation, the following apparatus was constructed. Metal tubes, of approximately 1 mm. bore, were soldered into holes made in the bottom of a small metal box (10 mm. long by 8 mm. wide by 4 mm. deep). An air-inlet tube was soldered into the side of the box. The latter was then sealed by means of its lid.

Air delivered by these jets serves two main purposes. First, water vapor is blown from the tubes, thus aiding drying, and secondly, bubbles with adhering precipitate, which tend to escape, are broken so that a loss of material is prevented. The air should be filtered and its rate of flow should not be great enough to cause loss of spray.

(d) Ashing

A constant ashing temperature is necessary in order to obtain reproducible results, since the apparent PBI value increases within limits with rising temperature. A temperature of 640° ± 10° C. for one and a half to two hours was found to be satisfactory.

(e) Dissolving the Ash

It is desirable to separate the resulting solution from the insoluble portion of the ash (e.g. carbon flakes) in order to prevent the particles from clogging the pipette in subsequent operations and from clouding the colorimetric solution. The best method for achieving this is to pour the supernatant carefully from the centrifuged sample into a second tube.

(f) Colorimetry

Since the colorimetric reaction is fairly temperature-sensitive, it is necessary to control this variable within close limits. A temperature of 39.1° ± 0.1° C. was found to be satisfactory.

(g) Curve of Standards

Various methods have been employed in the preparation of the curve of standards. Some investigators have simply used solutions of sodium iodide for this purpose. However, in the precipitation of the protein-bound iodine, some of the precipitating agents become bound to the plasma proteins and are carried through subsequent operations where they later interfere with the colorimetric reaction. It has been shown conclusively by Acland (3) that zinc from the zinc hydroxide precipitate inhibits the catalytic action of iodide on the reduction of ceric ion and he takes measures to ensure the absence of zinc from the final reaction. With the 6.7% albumin solution used to simulate the plasma proteins, such a standard curve was found to lie below a similar one prepared solely from solutions of sodium iodide. In other words, the use of the latter gives higher values for the protein-bound iodine. This is in agreement with the results of Barker *et al.* (1). Our data indicate that for a plasma having a content of 6 μg.% protein-bound iodine, for example, use of a simple calibration curve would yield a value that was 1.8 μg.% high. At more elevated levels, the discrepancy was somewhat smaller but at lower levels it was larger than the figure quoted.

The following experiment showed that the egg albumin did not contain any protein-bound iodine. To samples of a given plasma, ranging from 0.1 to 0.5 ml., was added sufficient albumin solution to bring the final volume up to 0.5 ml. (the volume of plasma usually taken). Analysis of these samples indicated that the egg albumin was not the source of any protein-bound

iodine. These results likewise suggested that the plasma and egg albumin solution behaved in a similar fashion with respect to any ultimate effect on the colorimetric reaction.

Recovery Experiments

The recovery of iodine, added as sodium iodide, is shown in Table II. In two sets of experiments, a known amount of iodide was added to plasma of previously determined PBI concentration (5.2 μg.%) just prior to the drying of the precipitated protein. As indicated the recovery was 95.0–96.5%.

Normal Range of Values

Estimations of the protein-bound iodine of 95 healthy subjects fell, for the most part, within the range 3.4 to 7.9 μg.%. There were no values greater than 8.0 while there was one value of 3.2. The limits indicated above are similar to those (3.5–8.0) reported by Barker *et al.* (1) using their method, which does not depend on the use of a calibration curve.

TABLE II

RECOVERY OF ADDED IODIDE

No. of aliquots	Iodide added, μg.%	Iodide recovered, μg.%	Percentage recovery	S.E.M., %
8	6.0	5.8	96.5	±1.2
6	6.0	5.7	95.0	±1.4

Acknowledgment

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References

1. BARKER, S. B., HUMPHREY, M. J., and SOLEY, M. H. J. Clin. Invest. **30**, 55 (1951).
2. SALTER, W. T. and MCKAY, E. A. Endocrinology, **35**, 380 (1944).
3. ACLAND, J. D. Biochem. J. **66**, 177 (1957).

PARTITION CHROMATOGRAPHY OF ORGANIC ACIDS: VARIATIONS IN SOLVENT COMPOSITION[1]

R. P. HARPUR

Abstract

In order to facilitate the analysis of nonvolatile organic acids by partition chromatography with silica, the relation of solvent composition to the rate of movement of the acids has been investigated. It was found that with chloroform, *n*-amyl alcohol, and dilute sulphuric acid the logarithm of the peak volume, and hence of the partition coefficient, is proportional to the logarithm of the alcohol concentration. The spread of the zones bears a direct linear relation to the peak volume. A simple graphical method is shown for planning a suitable solvent program for any particular set of acids. Stepwise elution is more suitable than a logarithmic gradient for this type of system.

Introduction

Several methods have been described for the analysis of organic acids by partition chromatography with silica or celite (2, 3, 6, 7, 14, 18). Sulphuric acid is used as the stationary phase and chloroform with different alcohol mixtures is commonly employed for the mobile phase. To cover the range of acids desired it has been necessary to use either stepwise or gradual changes in solvent composition. As the partition coefficients change with a change in solvent composition the conventional methods for expressing the rate of movement of the acids in relation to the movement of the solvent have not been used. Each method defines a certain solvent program and shows the separation of the mixture of acids in which the authors were interested. If one wishes to extend any particular method to include the separation of another acid, the only available procedure appears to be that of trial and error.

Marshall *et al.* (11) used the total volume of polar solvent required for elution to the peak volume to define the relation of each acid to the solvent system. Unfortunately, as will be shown later, the rate of movement of a solute down the column is not directly proportional to the alcohol concentration. Thus, when these authors used relatively high alcohol concentrations there was a change in the "alcohol volume". Mader (9) related a logarithmic rise in polar solvent concentration to the peak volumes and suggested that the equations he derived could be used to characterize the acids. Unfortunately this concept has not been extended to other solvent programs.

It is evident that some simple method of describing the rate of movement of each acid down a column is required whether it be for a stepwise or a gradual change in the composition of the eluting solvent.

Alm, Williams, and Tiselius (1) and Drake (4) have outlined the theory of gradient elution. They pointed out that this type of elution will not improve the separation over that of simpler elution techniques, when the isotherms are linear, that is, in the case of partition chromatography, when the partition

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coefficients are independent of the concentrations of the solutes. It will be obvious to anyone making a comparison of the different methods for the organic acids that not only must the peak volume be defined but so also must be the amount of spread of the zone.

Finally, it may be noted that only logarithmic gradients have been used so far for the elution of organic acids. These gradients have a serious disadvantage. For instance, the gradient which is small enough to allow the separation of lactic and succinic acids may not be large enough for the convenient elution of such acids as citric and tartaric (11) and vice versa (8). In only one case has a separation of lactic and succinic acids been combined with the elution of citric acid (17). Thus, if a gradient is to have any advantage in the elution of organic acids, this problem must be taken into account.

The purpose of this paper is not so much to present the best method for the analysis of the organic acids but rather to demonstrate a procedure for making the best use of a particular solvent system. Acids of interest in plant and animal biochemistry were selected for study.

Materials and Methods

The ambient temperature was 20–25° C.

Preparation of the column.—Two grams of silica, 100 mesh (Mallinckrodt), were ground with 0.06 *N* sulphuric acid. The acid was added in the largest quantity (1.3 ml. for the batches of silica used in this work) that would still give a smooth slurry with chloroform. Chloroform was added in 5 ml. portions until the slurry was thin enough for pouring. The slurry was packed with a disk of filter paper and a glass plunger into a tube of 1 cm.^{2} cross section (16). The height of the column before the addition of the sample was 4 cm. and the volume of mobile solvent in the column at any one instant was 1.6 ml., as measured by the passage of Sudan III dissolved in chloroform. The column was equilibrated with the required solvent prior to the addition of the acids.

Addition of acids.—Aqueous solutions of the acids were made alkaline to phenol red and evaporated to dryness on a steam bath. Compounds suspected of instability under these conditions, such as sodium pyruvate and oxalate, α-ketoglutaric, *cis*-aconitic, and tartaric acids, were weighed and added at this point. The residue was dissolved by grinding with 0.05 ml. of 0.3 *N* H_2SO_4. Additions of 0.001 ml. of 5 *N* H_2SO_4 were made until the indicator changed from yellow through orange to a definite pink color and finally the solution was diluted to a volume of 0.15 ml. with 0.3 *N* H_2SO_4. Silica, 0.2 g., was mixed with the solution and the mixture added to the top of the column after the filter paper and excess solvent had been removed. One milliliter of solvent was added, the silica was mixed with it by shaking, and the column packed with a disk of filter paper and a glass plunger. The plunger was washed with a further 1 ml. of solvent and the solvent was forced into the column before further additions. A pressure of 3–5 lb./in.^{2} was used to maintain a flow rate of 1 ml./minute.

Solvents.—Chloroform, U.S.P., was washed with dilute alkali, distilled, and dried over calcium chloride shortly before use. It was recovered by the same technique. The specific gravity was always greater than 1.487. Solutions of chloroform, *n*-amyl alcohol (reagent grade), and water were prepared in the following manner. Mixtures up to 11% (v/v) *n*-amyl alcohol in chloroform (CA_{11}) were shaken with 10% (v/v) of water and then filtered through filter paper. For mixtures of 12–60% of alcohol a measured amount of water was dissolved in the *n*-amyl alcohol with the help of a magnetic stirrer, and the solution diluted to volume with chloroform. If too much water was added the solution became cloudy. The amount of water required can be calculated with the following empirical formula:

$$\%\ \text{water (v/v final mixture)} = 0.01\ (\%\ n\text{-amyl alcohol})^{1.36}.$$

Detection of acids.—The eluate was collected in 1 or 2 ml. portions with a fraction collector, care being taken to avoid evaporation losses (10). The fractions were titrated by the monophasic system previously described (5). With the small volumes used here only one-half minute of gassing was required prior to titration.

Results

Relation of Solute Concentration to Peak Volume

Preliminary experiments, with constant solvent composition, indicated that with the exception of oxalic acid and possibly acetic acid, increasing solute concentration did not decrease the peak volumes. Table I shows some of the

TABLE I

EFFECTS OF EXCESS SOLUTE ON PEAK VOLUME

Acid	Solvent	Amount, μeq.	Peak volume, ml.
Acetic	CA_6	4	13.5
		20	13.0
		40	13.5
	CA_3	4	10.5
		40	8.5
Fumaric	CA_6	8	38
		20	39
		40	44
		80	49
Lactic	CA_6	4	85
		20	85
		40	86
Succinic	CA_6	8	155
		20	155
		40	158
		80	161
Pyruvic	CA_5	6	30.5
		20	30.5
Oxalic	CA_{40}	5	30
		18	23
		40	22
Citric	CA_{50}	4	50
		20	50
		40	50

acids tried and shows also that if more than 20 microequivalents of an acid are added there is sometimes an increase in peak volume rather than a decrease. This may be regarded as an overloading of the column. Below this limit an analogy may be made to adsorption chromatography and the isotherms considered to be linear within the region of 4–20 microequivalents (cf. 20). Furthermore, the elution curves are symmetrical. Again oxalic acid is an exception and tails badly; its quantitative analysis by means of the present method is not recommended. The adsorption properties of commercial silica and the suppression of the ionization of oxalic acid have been studied recently by Raveux and Bové (19).

Relation of Alcohol Concentration to Peak Volume

When the solvent composition was kept constant throughout each individual experiment it was found that the logarithm of the peak volume of any one acid is directly proportional to the logarithm of the concentration of *n*-amyl alcohol in the solvent. The curves relating peak volume to solvent composition for some of the acids investigated are shown in Fig. 1. *cis*-Aconitic and oxalic acids are included in Fig. 1 but since both gave low recoveries it is not

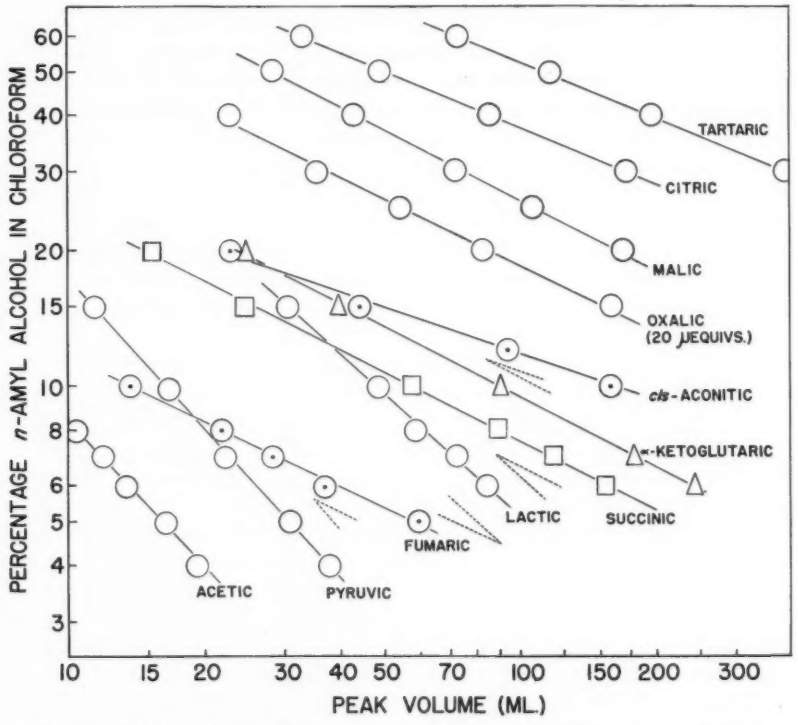

FIG. 1. Relation of peak volume to *n*-amyl alcohol concentration (see preparation of column for dimensions).

recommended that they be analyzed by this particular method. The curves for glyoxylic and acetic acids are very similar, and also those for malonic and α-ketoglutaric acids. During the summer months (temperature 27–30° C.) it was found that the acids moved more slowly. This temperature effect has not been investigated further.

Spread of the Solutes

In order to obtain a simple method for an approximate definition of the spread of the elution curves, two suggestions have been taken from the theoretical treatments (12, 15). The first is the approximation of the elution curves in partition chromatography to the normal curve of error and the other is the use of probability paper.

When the cumulative titration values for each acid were plotted on probability paper against the volume of eluate, slightly curved lines were obtained. Since the titration errors were relatively large at either end of the elution curves, only the center portions of the probability curves were used. The 50% value corresponded closely to the peak volume. The number of milliliters to include 34% of the total titration value immediately before the peak was always less than the number to include 34% after the peak. These values are arbitrarily called a "deviation" before the peak and a "deviation" after the peak. In Figs. 2 and 3 the "deviations" for all the different acids are plotted against the peak volumes. The lines were obtained by the method of least squares. Unfortunately, there were insufficient data to determine the lines for each acid with any accuracy but for practical purposes the information obtained from Figs. 2 and 3 appears to be satisfactory. They confirm the previous observation of Martin and Synge (13) with a partition system and show that there is a linear relation between the peak volume and the amount of spread.

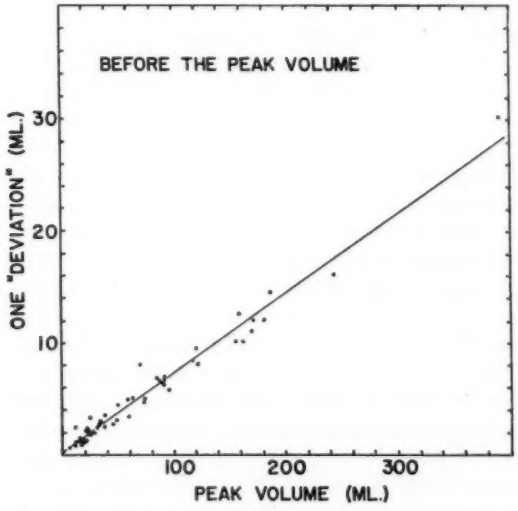

FIG. 2. Relation of the spread before the peak to peak volume.

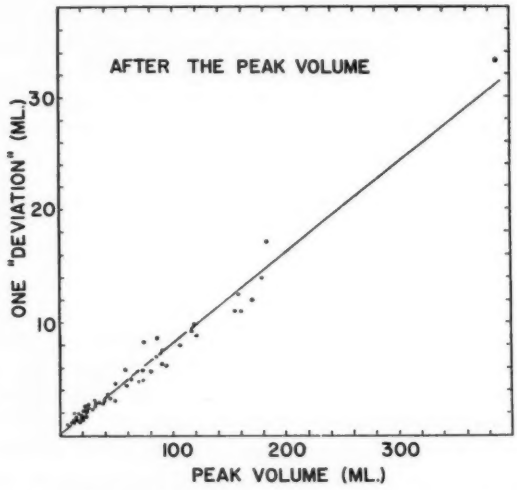

FIG. 3. Relation of the spread after the peak to peak volume.

Prediction of Peak Volumes and Spread

If the assumption is made that three "deviations" before and three "deviations" after the peak will include the volume of eluate necessary for the quantitative collection of each acid, it is a simple matter to draw lines on Fig. 1 parallel to the peak lines at a distance of three "deviations" on each side and thereby to show the spread of each acid at the given alcohol concentration. At alcohol concentrations where the spreads of adjacent pairs do not overlap, complete separation will be obtained. The dotted lines in Fig. 1 indicate the more critical areas. Succinic and α-ketoglutaric acids cannot be separated completely at any alcohol concentration. From this composite graph it is easy to determine a suitable solvent program for any particular set of separations. Drake's (4) graphical method which may be used for stepwise or gradient elution, is useful in portraying the movement of the bands down the column. An example is shown in Fig. 4. The upper part of the figure shows the solvent program, the middle part the movement of the bands, and the lower part shows some titration values actually obtained. In the middle part the continuous lines represent the movement of the peaks. The rate of movement or slope of these lines is obtained from Fig. 1 for the appropriate alcohol concentration. In the same way the dotted lines represent three "deviations" from the peak at the appropriate rate of movement. The width of the band is represented horizontally in terms of the volume of eluate which would be required to elute the band if the column were cut at that level. When the solvent is changed it is assumed that the leading and tailing edges may be treated as separate peaks which will then deviate forward and backward respectively, in the same manner as the main peaks. The results actually obtained are in good agreement with those predicted. Experiments with 1-g.

and 4-g. columns instead of the usual 2-g. column showed that the velocity of the acids was constant and that, for the approximate calculation of the peak volumes for different-sized columns, the silica used for adding the acids could be neglected.

It will be noted from Fig. 4 that it is also possible to obtain a separation with a 1-g. column. This figure shows, however, the compression in band width which can be obtained with some of the acids by the use of stepwise elution.

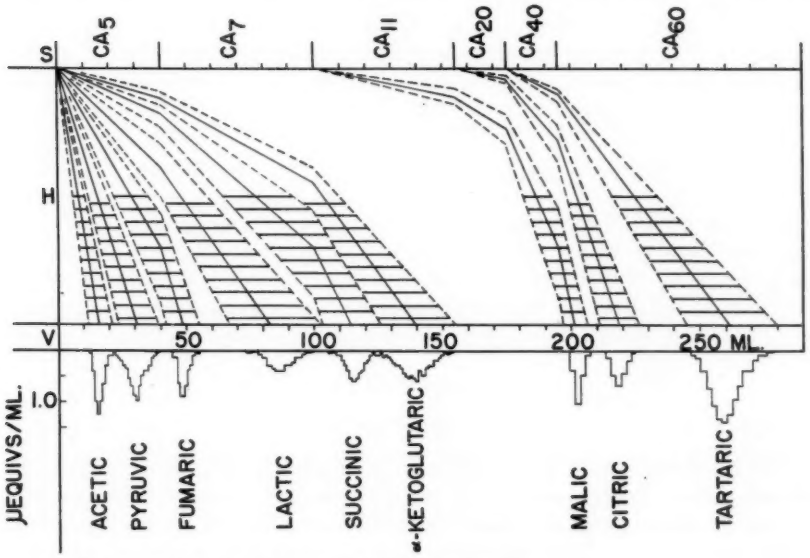

FIG. 4. Migration of acid bands down the column.
S = solvent program.
H = height of the column (see preparation of column for dimensions).
V = volume of eluate.

Discussion

Preliminary studies with *n*-butyl, *n*-amyl, and *tert*-amyl alcohols indicated that the relation shown in Fig. 1 existed, and that either of the last two alcohols would allow the separation of lactic and succinic acids. From this information *n*-amyl alcohol was selected for the more detailed study. Mixtures of the alcohols were not tried, nor was the concentration of the sulphuric acid in the column varied.

It is possible that the log–log relation between solvent composition and peak volume is a general property of this type of system. If such is the case the preliminary experiments for developing a method should be greatly facilitated since the peak volumes could be anticipated by extrapolation. When the stationary phase is a relatively high concentration of sulphuric acid, it is not easy to determine the partition coefficients of organic acids directly. For practical purposes, the peak volume is commonly used to describe the relation

of a solute to a particular chromatographic column and solvent system. In order to compare the efficiency of solvent systems for any particular separation and without regard to column dimensions or solvent programs, it is suggested that the partition coefficients should be used to define the behavior of a solute in a solvent system. Table II shows the relation of the solvent concentration to the partition coefficients (calculated from the formula of Martin and Synge (12)).

TABLE II

RELATION OF PARTITION COEFFICIENT (α) TO *n*-AMYL ALCOHOL CONCENTRATION (C)

(Within the limits shown in Fig. 1)

Acid	Log α
Pyruvic	2.04−0.99 log C
Acetic	1.76−1.02 log C
Lactic	2.68−1.12 log C
α-Ketoglutaric	3.79−1.96 log C
Malic	4.64−1.96 log C
Succinic	3.67−2.05 log C
Fumaric	3.24−2.27 log C
Citric	5.73−2.44 log C
Tartaric	6.08−2.44 log C
(*cis*-Aconitic	5.04−2.96 log C)

The determination of the spread can be carried out concurrently with the determination of peak volumes and it is sufficiently accurate for the practical purpose of determining the most suitable solvent program for the separation of any given acids. Since the tailing edge has more time to deviate than the leading edge, it is expected that the "deviation" after the peak will be larger than that before the peak.

According to the theory of Alm *et al.* (1) the term gradient should be applied not so much to the inflowing solvent as to the difference in solvent composition at any instant between the leading and tailing edge of the band. In the center part of Fig. 4 the scale for the height of the column is much larger than that for the volume of eluate. This is for clarity in presentation and for practical considerations in graphically computing the movement of the solutes. However, the height of the column, after the addition of the acids, represents only 1.75 ml. of mobile solvent and if the band were spread over even as much as half the height of the column it would represent only 0.9 ml. of mobile solvent. Now it can be seen from Fig. 1 that a change of at least one per cent in the alcohol concentration is required to make any appreciable difference in the rate of movement. Thus the gradient required to cause any compression of the bands by gradient alone would need to rise from CA_0 to CA_{60} in at least 54 ml. Thus, with the slope of solvent change which would allow as complete a separation as possible of, for example, lactic and succinic acids, one would expect little compression of the bands.

Even with very steep concave gradients it was not possible to decrease the tailing of oxalic acid any more than could be obtained by elution at constant solvent composition equal to that at which the gradient finally eluted the acid. Similarly, with other acids no detectable compression of the bands could be obtained by a gradient other than could be obtained by a stepwise elution.

A second point in the theory of gradient elution is that the gradient removes the risk of false peaks which can occur with a stepwise elution. Again, this applies to those systems where there is bad tailing of the bands. The only false peak which can occur, with the exception of oxalic acid or an acid undergoing decomposition on the column, occurs when an unexpected acid is eluted in the midst of a solvent change. In such a case the solvent program would require rearrangement.

The data presented here indicate clearly that a gradual increase in the alcohol content of the chloroform is not necessary for the separation of the organic acids by partition chromatography. With samples likely to contain unexpected acids, a gradation from one step to another would be an advantage. However, with known mixtures a satisfactory separation may be obtained by using steps arranged so that each acid is eluted from the column at the fastest rate that does not cause contamination with the adjacent acids.

Acknowledgments

Appreciation is expressed for the technical assistance of Mrs. J. Carter. The financial assistance of the National Research Council of Canada is gratefully acknowledged.

References

1. Alm, R. S., Williams, R. J. P., and Tiselius, A. Acta Chem. Scand. **6**, 826 (1952).
2. Bulen, W. A., Varner, J. E., and Burrell, R. C. Anal. Chem. **24**, 187 (1952).
3. Donaldson, K. O., Tulane, V. J., and Marshall, L. M. Anal. Chem. **24**, 185 (1952).
4. Drake, B. Arkiv Kemi, **8**, 1 (1955).
5. Fairbairn, D. and Harpur, R. P. Can. J. Chem. **29**, 633 (1951).
6. Frohman, C. E., Orten, J. M., and Smith, A. H. J. Biol. Chem. **193**, 277 (1951).
7. Isherwood, F. A. Biochem. J. **40**, 688 (1946).
8. Ladd, J. N. and Nossal, P. M. Australian J. Exptl. Biol. Med. Sci. **32**, 523 (1954).
9. Mader, C. Anal. Chem. **26**, 566 (1954).
10. Mader, C. and Mader, G. Anal. Chem. **25**, 1556 (1953).
11. Marshall, L. M., Donaldson, K. O., and Freidberg, F. Anal. Chem. **24**, 773 (1952).
12. Martin, A. J. P. and Synge, R. L. M. Biochem. J. **35**, 1358 (1941).
13. Martin, A. J. P. and Synge, R. L. M. Biochem. J. **35**, 1364 (1941).
14. Marvel, C. S. and Rands, R. D. J. Am. Chem. Soc. **72**, 2642 (1950).
15. Mayer, S. W. and Tompkins, E. R. J. Am. Chem. Soc. **69**, 2866 (1947).
16. Neish, A. C. Can. J. Research, **28B**, 535 (1950).
17. Pennington, R. J. and Sutherland, T. M. Biochem. J. **63**, 618 (1956).
18. Phares, E. F., Mosbach, E. H., Denison, F. W., Jr., and Carson, S. F. Anal. Chem. **24**, 660 (1952).
19. Raveux, R. and Bové, J. Bull. soc. chim. France, 369 (1957).
20. Williams, R. J. P., Hagdahl, L., and Tiselius, A. Arkiv Kemi, **7**, 1 (1954).

PRODUCTION WITH SODIUM SULPHATES OF AN ELECTROLYTE–STEROID-CARDIOPATHY CHARACTERIZED BY NECROSES (ESCN), AND ITS PREVENTION BY $MgCl_2$ AND KCl[1]

HANS SELYE

Abstract

Massive myocardial necroses and hemorrhagic necroses of the liver can be produced by combined treatment with monosodium or disodium sulphate ($NaHSO_4$ or Na_2SO_4) and 2α-methyl-9α-chlorocortisol (Me-Cl-COL) in the rat. In this respect, the effect of the sulphates resembles that previously described for sodium phosphates and perchlorate. It differs from that of most other sodium salts that have been tested for this activity up to the present time.

NaCl slightly inhibits the cardiotoxic and hepatotoxic effect of concurrent treatment with Na_2SO_4 plus Me-Cl-COL. Equimolecular amounts of $MgCl_2$ or KCl are even considerably more effective in this respect. It is noteworthy that, under these experimental conditions, the effect of 1 mM. of Na_2SO_4 can be inhibited by 0.5 mM. of $MgCl_2$ or KCl.

We may conclude from these observations that, in rats conditioned by a highly active corticoid, such as Me-Cl-COL, the production of an "infarctoid cardiopathy" (as well as of the accompanying hepatic changes) does not only depend upon the sodium ion, but is largely influenced by the simultaneous presence of other anions and cations.

Introduction

The electrolyte–steroid-cardiopathy characterized by infarct-like myocardial necroses (ESCN) has been described at length elsewhere (1, 2). Let us merely recapitulate here that, in animals conditioned with certain highly active corticoids, such as 2α-methyl-9α-chlorocortisol (Me-Cl-COL), simultaneous treatment with some, but not all, sodium salts elicits widespread myocardial necroses. These are often accompanied by hepatic necroses and nephrocalcinosis.

The object of this communication is to describe experiments showing that both monosodium and disodium sulphate are highly effective in this respect and that their toxic actions in Me-Cl-COL-treated rats can be inhibited by concurrent administration of either $MgCl_2$ or KCl.

Experimental

One hundred female Sprague–Dawley rats, with a mean initial body weight of 102 g. (range: 90–111 g.), were subdivided into 10 equal groups and treated as indicated in Table I.

2α-Methyl-9α-chlorocortisol (Me-Cl-COL) was administered in the form of a microcrystal suspension of its acetate, at the daily dose of 100 μg. in 0.2 ml. of water, subcutaneously.

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All electrolytes were given by stomach tube, twice daily, the individual dose of each salt or salt mixture being dissolved in 5 ml. of water. Each single dose of monosodium sulphate ($NaHSO_4.H_2O$), disodium sulphate (Na_2SO_4), and the sodium chloride (NaCl) in Group 4, corresponded to 1 mM.; in addition, 0.5 mM. of NaCl, magnesium chloride ($MgCl_2.6H_2O$), or potassium chloride (KCl) was administered to the rats of Groups 8, 9, and 10 respectively. Preliminary experiments had shown that the cardiotoxic effects of 1 mM. of the sodium sulphates can be inhibited by 0.5 mM. of magnesium or potassium chloride, so that it was not thought necessary to overburden the animals with 1 mM. of each of these electrolytes; NaCl was given for a comparison, at the same low dose level.

Throughout the experiment, the animals were kept exclusively on "Purina Fox Chow" (of the Ralston Purina Company of Canada) and tap water. After 12 days of treatment, the survivors of all groups were killed with chloroform. However, by that time, several of the animals treated with sulphates plus Me-Cl-COL had died, exhibiting macroscopically visible, large patches of myocardial necroses, as well as multiple hemorrhagic and necrotic patches in the liver. Hence, immediately after autopsy, the hearts and livers of the survivors were fixed in Susa solution for the subsequent staining of paraffin-embedded sections with hematoxylin–phloxine.

Table I lists the mean intensity of the cardiac and hepatic necroses (expressed in terms of an arbitrary scale of grade 1 to 3) with standard errors, as well as the percentual mortality rate.

Results

Perusal of Table I indicates that, in rats treated with $NaHSO_4$ (Group 1), Na_2SO_4 (Group 2), Me-Cl-COL (Group 3), NaCl (Group 4), or NaCl plus Me-Cl-COL (Group 5), no morphologic changes were induced either in the heart or in the liver, nor was there any mortality. On the other hand, among

TABLE I

RESULTS OF COMBINED STEROID–ELECTROLYTE TREATMENT

Group	Treatment	Necroses (scale: 1 to 3)		Mortality, %
		Cardiac	Hepatic	
1	$NaHSO_4$	0	0	0
2	Na_2SO_4	0	0	0
3	Me-Cl-COL	0	0	0
4	NaCl	0	0	0
5	NaCl+Me-Cl-COL	0	0	0
6	$NaHSO_4$+Me-Cl-COL	1.1±0.4	0.5±0.3	50
7	Na_2SO_4+Me-Cl-COL	2.1±0.35	1.3±0.45	80
8	Na_2SO_4+Me-Cl-COL+NaCl	0.4±0.22	0	20
9	Na_2SO_4+Me-Cl-COL+$MgCl_2$	0	0	0
10	Na_2SO_4+Me-Cl-COL+KCl	0	0	0

the rats treated with either $NaHSO_4$ or Na_2SO_4, in combination with Me-Cl-COL (Groups 6 and 7), there was a high mortality and the cardiac as well as the hepatic necroses were severe. The fact that, in these respects, Na_2SO_4 was more effective than $NaHSO_4$ is consonant with our earlier observations that showed that, among the phosphates, Na_2HPO_4 is more effective than NaH_2PO_4 after similar conditioning with Me-Cl-COL.

The mortality rate and organ changes were only slightly reduced, in the rats treated with Na_2SO_4 plus Me-Cl-COL, by the additional administration of NaCl (Group 8), but completely inhibited by $MgCl_2$ (Group 9) and KCl (Group 10). The prophylactic effect of all three of these chlorides proved to be statistically highly significant ($P <$ than 0.01).

Acknowledgment

The author wishes to gratefully acknowledge 2α-methyl-9α-chlorocortisol (Me-Cl-COL), supplied by The Upjohn Company.

References

1. SELYE, H. Brit. Med. J. March 15, 599 (1958).
2. SELYE, H. The chemical prevention of cardiac necrosis. The Ronald Press Co., New York. 1958.

ANAEROBIC GLYCOLYSIS IN BRAIN SLICES AFTER DEPRIVATION OF OXYGEN AND GLUCOSE[1]

K. A. C. Elliott and M. Rosenfeld

Abstract

If brain slices are deprived of oxygen and glucose while they are incubated in saline medium at 38° C. the respiratory activity, subsequently determined in the presence of oxygen and glucose, is slowly decreased; the anaerobic glycolytic activity, determined after adding glucose, is very rapidly lost. These effects are prevented if the deprivation occurs at 0° C.

Loss of glycolytic activity does not occur rapidly if the brain is kept in the head at 38° after decapitation nor if slices are kept humid but without suspending medium at 38° in the absence of oxygen.

The glycolytic activity of slices which have been deprived of oxygen and glucose in saline medium at 38° can be largely restored by a period of aerobiosis or by merely replacing the medium with fresh oxygenated medium.

Addition of pyruvate to the medium stimulates glycolysis by normal slices but not that by deprived slices. The stimulatory effect of pyruvate reappears if the deprived slices are given a period of aerobiosis.

It is concluded that the drastic irreversible effects of brief cerebral anemia on brain function cannot be ascribed to irreversible loss of aerobic or anaerobic metabolic activity.

Introduction

The anaerobic glycolytic activity of brain slices is extremely variable. Rates varying from 3.5 to 25 μl. of carbon dioxide liberated from bicarbonate-buffered medium per milligram dry weight per hour have been reported for glycolysis by rat cerebral cortex slices under apparently similar conditions. Glycolysis is normally inhibited in the presence of oxygen but with brain this inhibition takes a variable time to set in. A large number of other agents affect glycolysis; some simply inhibit it, some cause an increase in aerobic glycolysis without affecting anaerobic glycolysis, and others increase aerobic glycolysis and inhibit anaerobic glycolysis. Among the conditions which affect glycolysis by slices are some which might have considerable significance in vivo. A period of aerobiosis causes a marked increase in the rate of glycolysis found during subsequent anaerobiosis. Pyruvate added to the suspending medium also stimulates. Elevated potassium concentration, in the medium, addition of glutamate or ammonia, or application of electric pulses to the tissue, stimulates aerobic and inhibits anaerobic glycolysis. References to all the above observations are given in reference 1.

Dickens and Greville (2) found that incubation of brain slices in the absence of both oxygen and glucose caused some loss of their ability to consume oxygen when oxygen and glucose were subsequently supplied. The anaerobic glycolytic activity was, however, much more strongly depressed. The probable importance of the glycolytic ability of brain in vivo has been discussed elsewhere (1, 3). It seemed possible that the rapid irreversible damage which

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is done to the physiological activity of the brain in vivo by brief periods of interference with circulation might be related to loss of glycolytic activity. The nature of the effect of deprivation of oxygen and substrate on subsequent glycolysis in vitro has therefore been more closely studied.[2]

Methods

Adult rats were decapitated with a guillotine, the brains were excised, and slices of cerebral cortex were cut in a humid chamber with a Stadie–Riggs microtome. The slices were kept in the humid chamber until they were weighed and introduced into Warburg vessels. For studies on glycolysis the vessels contained 3 ml. of saline medium of the following composition in millimoles per liter: NaCl 122, KCl 3.1, $MgSO_4$ 1.2, $CaCl_2$ 1.3, KH_2PO_4 0.4, and $NaHCO_3$ 24.5. For respiration measurements the bicarbonate was replaced by sodium phosphate 17 mM. per liter, pH 7.6, and calcium was omitted. Glucose, final concentration 10 m*M*, was added from a side arm at the time indicated for the particular experiment. The vessels containing slices and medium were kept in a tray of ice until they were filled with gas and put in the bath. (This was necessary since, in some experiments, some of the vessels had to be kept waiting for variable times. As shown below, changes in the metabolic activity of the tissue are minimized by cold.) The vessels were filled with oxygen (for respiration) or nitrogen – 5% carbon dioxide (for glycolysis), and respiration or glycolysis was measured manometrically at 38° by the standard Warburg procedure. Further details and modifications of the above procedure are described in the legends to figures and tables.

Results

The results illustrated in all figures are representative of two or more similar experiments.

In Figs. 1 and 2 examples are shown of the effects of periods of deprivation of oxygen and glucose at 38° C. on subsequent respiratory and glycolytic activity of brain cortex slices in the presence of glucose. In confirmation of previous work (2) it was found that respiratory activity was depressed slowly by previous deprivation of oxygen or glucose and somewhat more rapidly by deprivation of both. In the absence of oxygen, but with glucose present, glycolysis was active initially but fell off with time. A period of deprivation of both oxygen and glucose caused very rapid loss of glycolytic capacity; effects were observable after 3 minutes and inhibition was nearly complete after 30 minutes of deprivation.

Effects of Physical Factors

As shown in Figs. 3 and 4 the effects of deprivation on the subsequent respiration and anaerobic glycolysis by brain slices are greatly reduced if the

[2]Preliminary work on this subject was carried out by Mrs. Jean Cross Silva, who showed that many of the observations now reported can be made with suspensions of brain prepared in isotonic saline media and who demonstrated the effect of cold in preserving glycolytic activity (4).

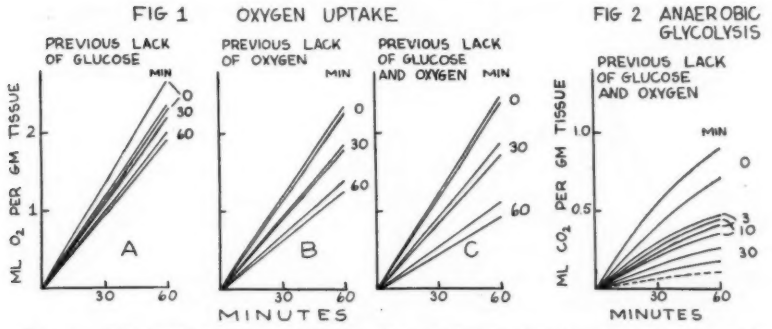


FIG. 1. Effects of incubation at 38° in saline medium in the absence of glucose and/or oxygen on the subsequent respiration.

A. Slices were incubated aerobically. Glucose was added to the medium of different vessels initially, after 30 minutes, and after 60 minutes. Readings were begun after the last addition.

B. Glucose was present in the medium throughout in all vessels. One pair of vessels contained oxygen throughout; the others were filled with nitrogen and, after 30 or 60 minutes, this gas was replaced with oxygen. Readings were begun after equilibration following the last gas change.

C. Glucose and oxygen were present throughout in one pair of vessels. The other vessels were filled with nitrogen and the medium lacked glucose initially; after 30 or 60 minutes glucose was added and the vessels were filled with oxygen. Readings were begun after equilibration following the last gas change.

FIG. 2. Effects of incubation at 38° in saline medium in the absence of oxygen and glucose on subsequent anaerobic glycolysis.

All vessels contained N_2/CO_2 throughout. Glucose was tipped into the medium in two vessels before incubation and at 3, 10, or 30 minutes after the other vessels were placed in the bath. Readings for the first three pairs were begun 10 minutes after the vessels were placed in the bath and for the last pair immediately after glucose was added. The broken line shows readings obtained, in other experiments, in which no glucose was added.

temperature is kept low during the period of deprivation; the inhibitory effect can be nearly or completely prevented at 0°.

With suspensions it was found (4) that the damage to the glycolytic mechanism was increased appreciably by shaking, and considerably by dilution of the suspension, during the period of deprivation.

Possible Post-mortem Changes in Glycolytic Activity

The fact that loss of glycolytic activity could be prevented by lowering the temperature suggested a method for testing the possibility that a loss of activity may occur between the time of the death of the animal, or excision of the tissue, and the time when the tissue is immersed in glucose-containing medium. As is shown in Table I, respiratory and glycolytic activities within the usual range were found in tissue from brains which had been excised from the heads of rats 30 minutes after decapitation whether the head had been kept at 38°, room temperature, or chilled to about 0° C. during the 30 minutes. There was no sign that the period at 38° caused greater damage than at 0°.

It was also found (Table II) that, when slices were prepared immediately after decapitation and then kept anaerobically at 38° without medium, there was not a rapid loss of activity such as occurs when slices are immersed in glucose-free saline medium and incubated anaerobically.

FIG 3 OXYGEN UPTAKE

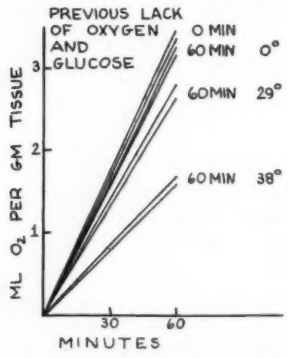


FIG 4 ANAEROBIC GLYCOLYSIS

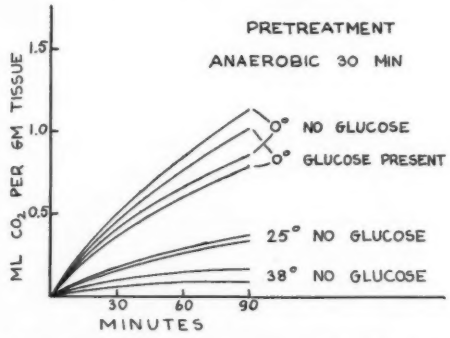


FIG. 3. Effect of temperature during previous deprivation of oxygen and glucose in saline medium on subsequent respiration.

One pair of vessels containing oxygen and glucose was shaken at 38° continuously. Others, filled with nitrogen and containing no glucose in the medium, were kept at 0° or shaken at 29° or 38°, for 60 minutes; glucose was then tipped in and the vessels were filled with oxygen and placed in the bath at 38°. Readings of all manometers were begun after temperature equilibration of the last vessels.

FIG. 4. Effect of temperature during previous deprivation of oxygen and glucose in saline medium on subsequent anaerobic glycolysis.

All vessels were filled with N_2/CO_2. One pair of vessels, with glucose in the medium, was kept at 0° for 30 minutes. Other vessels without glucose in the medium were kept for 30 minutes at 0°, 25°, and 38° and then glucose was tipped in. Readings were begun after all had been equilibrated at 38°.

TABLE I

EFFECTS OF LEAVING THE BRAIN IN THE HEAD FOR 30 MINUTES AFTER DECAPITATION

		Head kept at:				
		0°		21°		38°
		Tissue sliced at:				
		4°	21°	4°	21°	21°
Oxygen uptake, ml./g./hr.		2.6, 2.2	2.4	2.7, 2.5	2.4, 2.1	2.4, 2.5
Anaerobic glycolysis, ml./g./hr.	1st 30 min.	1.8, 1.5	1.6, 1.3	2.3, 2.5	2.3, 1.7	1.8, 1.5
	2nd 30 min.	0.8, 0.8	0.8, 0.6	1.4, 1.5	1.4, 1.0	1.0, 0.8

NOTE: Immediately after decapitation a head was immersed and held under mercury in a tube surrounded by ice and salt at −5° C. ("head kept at 0°"). Another head was left at room temperature and a third was kept in a tube immersed in the water bath at 38° C. The brains were dissected out and slices prepared in the cold room or in the ordinary laboratory.

One set of rats were used for the oxygen uptake, another for the glycolysis experiments.

TABLE II

EFFECT OF INCUBATING BRAIN SLICES ANAEROBICALLY AT 38° WITHOUT MEDIUM

Anaerobic glycolysis, ml./g./hr.

	Without medium, minutes				With medium lacking glucose, 30 min.
	0	30	60	90	
Subsequent anaerobic glycolysis (1st 30 min.)	1.5, 2.0	1.1, 1.3	1.6, 1.7	0.7, 0.8	0.1 to 0.4*

NOTE: Slices were placed in Warburg vessels containing some medium in the side arms (to maintain humidity) but none in the vessels. The vessels were filled with N_2/CO_2 and incubated for the times shown. Glucose-containing medium was then added, the vessels were regassed, and readings were taken.

*Range of numerous determinations.

The rapid loss of glycolytic activity on incubation in the absence of oxygen in medium lacking substrate is therefore in some manner connected with the medium in which the tissue is immersed.

Effect of Changing Medium

The glycolytic activity of slices, which had been apparently destroyed by brief incubation anaerobically in medium lacking glucose, partly reappeared if the medium was changed when glucose was introduced. The anaerobic glycolytic activity of normal slices commonly falls off after a variable time. If the suspending medium was removed and replaced with fresh medium after glycolysis had fallen off, the rate was restored to nearly the original level. If the rate had not yet fallen off, changing the medium had no obvious effect.

These results indicated that the major part of the glycolytic mechanism was not destroyed irreversibly during incubation in medium lacking oxygen and glucose. The possibility that accumulation of inhibitory substances could account for the above observations was excluded by experiments in which untreated slices were suspended in medium in which other slices had been incubated anaerobically with or without glucose. Such "used" medium caused only slight inhibition which could probably be accounted for by weak inhibitory effects of lactate and phosphate and other substances.

It thus seemed that the glycolytic activity observed was affected by some aspect of the manipulations involved in the experiments.

Effect of Previous Aerobiosis

It is well known that a period of incubation under aerobic conditions stimulates subsequent anaerobic glycolytic activity (5). With suspensions it has been found that, though full aerobiosis almost completely suppresses glycolysis, the rate of glycolysis is maximal when a trace of oxygen is present (6). It seemed possible that oxygen introduced with fresh medium might account for the restorative effect of changing the medium. In accordance with this, Fig. 5 shows that a marked stimulatory effect on glycolysis occurred if the fresh medium had been aerated with oxygen – carbon dioxide mixture but the effect was much less marked if the fresh medium had been freed of oxygen. (A little oxygen from the air would be dissolved in the latter medium during its introduction into the vessel.)

The effect evidently depends upon the temporary availability of a very little oxygen since the amount dissolved in the medium is small and much would be removed or consumed during the re-gassing of the vessels with oxygen-free gas and the subsequent 10 minute period of equilibration at 38° C. At this point a brief note by Quastel and Wheatley (7) came to our attention which stated that brain slices which had been made incapable of anaerobic glycolysis by previous exposure to nitrogen in the absence of glucose regained all their previous power of breaking down glucose anaerobically if they were exposed to oxygen and then to nitrogen and supplied with glucose. Results shown in Figs. 6 and 8 indicate that this is nearly true. The rate did not, however, reach that obtained with slices which had been stimulated by a

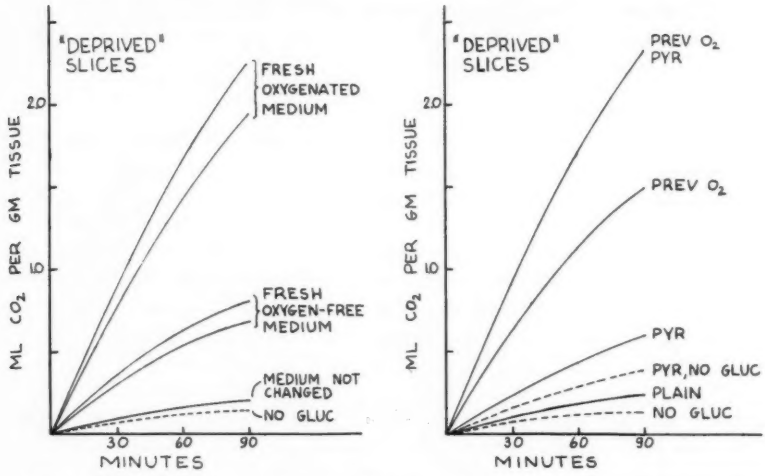


FIG. 5. Effect of changing the medium on anaerobic glycolysis by slices after anaerobic incubation in the absence of glucose.

Slices were incubated anaerobically at 38° in medium lacking glucose for 30 minutes. The medium was then withdrawn from two pairs of vessels and replaced with fresh glucose-containing medium which had been saturated with O_2/CO_2 or with N_2/CO_2. The vessels were then re-gassed with N_2/CO_2. The lower two curves represent average results in which, after the same pretreatment, the medium was not changed but glucose was added from the side arm or (broken line) no glucose added.

FIG. 6. Effects of pyruvate and a period of aerobiosis on anaerobic glycolysis by slices which have suffered incubation in the absence of oxygen and substrate.

All slices were incubated at 38° for 30 minutes anaerobically in medium containing no glucose. Glucose was then added from side arms to all *except* where indicated by *broken lines*.

Prev O_2: After the initial 30 minutes, vessels were briefly gassed with O_2/CO_2 and incubated for 20 minutes. They were then gassed with N_2/CO_2, equilibrated for 10 minutes, and readings were started.

Prev O_2 + Pyr: As "Prev Aerob" but pyruvate, 2 m*M* final concentration, was added immediately before the final gassing with N_2/CO_2.

Pyr: After the initial 30 minutes and addition of glucose incubation was continued for 20 minutes so that the total time and temperature conditions were the same as for the other vessels. Pyruvate was added from side arms 10 minutes before readings were begun.

Plain: As "Pyruvate" without addition of pyruvate.

Pyr, No Gluc, and No Gluc: The same as "Pyr" and "Plain" except that no glucose was added.

Each curve shown is the average of 4 to 10 determinations.

period of aerobiosis without having suffered a period of deprivation of oxygen and substrate. The rate achieved after a period of aerobic incubation was not greater than was obtained (Fig. 5) by simply changing the medium to freshly oxygenated medium.

Effect of Addition of Pyruvate

The addition of pyruvate to the medium is well known to accelerate anaerobic glycolysis (6, 8, 9, 10). Results of experiments on the effect of pyruvate are illustrated in Figs. 6, 7, and 8. The final concentration of pyruvate was 2 m*M*; the effect of 5 m*M* pyruvate was no greater.

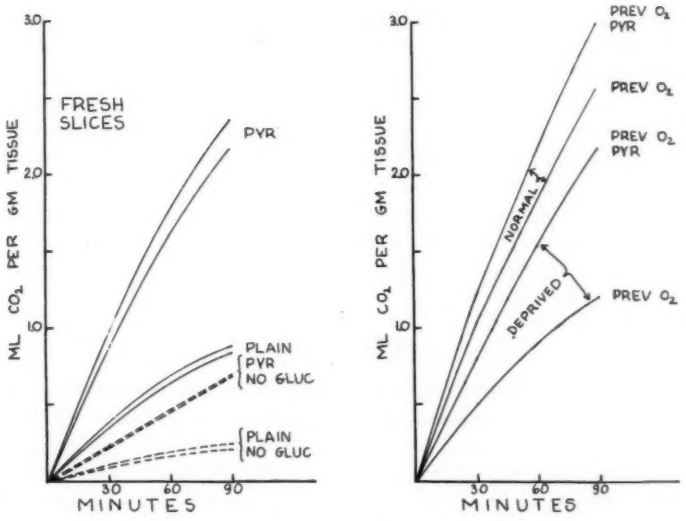


FIG. 7. Effect of pyruvate on glycolysis by fresh, untreated slices.
Before incubation glucose and pyruvate (2 m*M* final concentration) was added to one pair of vessels (Pyr), glucose alone to another pair (Plain), pyruvate alone to a third pair (Pyr, No gluc), and neither to the fourth pair (Plain, No gluc).

FIG. 8. Effect of deprivation of glucose and oxygen on subsequent anaerobic glycolysis stimulated by a period of aerobiosis and by pyruvate.
Slices were incubated anaerobically for 30–40 minutes in saline medium at 38°. The vessels were then gassed with O_2/CO_2 and incubated for 20 minutes, and then regassed with N_2/CO_2, and pyruvate, 2 m*M* final concentration, was added where indicated. After 10 minutes equilibration readings were begun.
Normal: Glucose present throughout.
Deprived: Glucose not added until after the initial anaerobic incubation.
Each curve shown is the average of four determinations.

With slices which had been incubated anaerobically without glucose, the addition of pyruvate together with glucose did not restore glycolytic activity (Fig. 6). (This fact was briefly noted by Dickens and Greville (2).) It will be seen in Fig. 6 that when pyruvate is added without glucose to such slices some evolution of carbon dioxide occurs. This can be accounted for by dismutation of pyruvate to lactate, acetate, and carbon dioxide (10, 11). When pyruvate was added with glucose to previously deprived tissue, the carbon dioxide evolution was little more than the sum of that due to slight residual glycolytic activity plus that due to the dismutation. On the other hand, with tissue which had not suffered previous deprivation the increase in CO_2 evolution on addition of pyruvate was definitely greater than could be accounted for by dismutation and was evidently the result of a marked stimulation of glycolysis (Fig. 7).

When previously deprived tissue was given a period of aerobiosis a stimulatory effect of added pyruvate in addition to that of previous aerobiosis was obtained (Fig. 6).

When both stimuli—a period of aerobiosis and addition of pyruvate to the medium—were applied to slices which have been incubated anaerobically with and without glucose, it was found that, while both sets of slices glycolyzed very actively, the slices which had suffered anaerobic incubation in the absence of glucose showed less activity than the slices which had not been so deprived (Fig. 8). The effects of deprivation are thus not completely reversible by applying the two stimuli. The extent of this irreversible damage to glycolytic activity, however, seems not to be appreciably greater than the damage done to the respiratory activity (Fig. 1C); both respiration and stimulated glycolysis are reduced by about 25% by incubation for 30 minutes in medium which lacks oxygen and glucose.

Discussion

Brain tissue suspended in saline medium at 38° in the absence of oxygen and glucose rapidly loses its glycolytic activity, but the activity can be largely restored by brief access to small amounts of oxygen. During the period of deprivation the enzymes involved in glycolysis are apparently not seriously irreversibly affected but some condition necessary for the initiation of the glycolytic process is changed. During anaerobiosis with glucose present from the start, active glycolysis proceeds and continues for a considerable time though the rate tends to fall. Evidently some of the initiating condition can be partially maintained during glycolytic activity. Brief access to oxygen restores the condition and, indeed, can bring it to a level which allows an increased rate of glycolysis.

It seems likely that the condition concerned is the concentration of high energy phosphate since adenosine triphosphate (ATP) is well known to be necessary for the initiation of glycolysis, to be produced liberally by oxidative phosphorylation during aerobic metabolism, and to be produced in less liberal amounts by glycolysis. It is readily destroyed by adenosine triphosphatases so that continuous production is required to balance the amounts destroyed or used in initiating metabolism. Macfarlane and Weil-Malherbe (12) found that the loss of glycolytic activity by rabbit brain slices on anaerobic incubation without glucose was accompanied by a decrease in the pyrophosphate P content of the slices. Studies are in progress to test whether the high energy phosphate or ATP content of the tissue can be correlated with its glycolytic activity following various treatments.

The fact that glycolytic ability is rapidly lost in the absence of oxygen and glucose only when the tissue is bathed in a suspending medium seems difficult to explain on the above hypothesis. It is possible, however, that ATP or substances from which it is derived may be leached out of the tissue by the suspending medium, or be more rapidly destroyed as a result of the changes in structure involved in the swelling which occurs in saline medium (13).

The mechanism of the stimulatory effect of adding pyruvate to the medium is evidently different from that of previous aerobiosis. Pappius *et al.* (14)

found conditions (substitution of lithium or potassium for all the sodium in the medium) under which pyruvate would stimulate glycolysis while previous aerobiosis would not. In the present study it is shown that glycolysis by tissue which has been previously deprived of oxygen and glucose is stimulated by brief aerobiosis but not by pyruvate. Pyruvate becomes effective, however, after brief aerobiosis.[3] It seems that pyruvate can exert its stimulatory effect only when the condition necessary for initiating glycolysis is present. A likely mechanism of the action of the added pyruvate is the effect of increased pyruvate concentration in driving reaction 1 to the right.

[1] $$\text{Pyruvate} + \text{DPN.H} \rightleftarrows \text{lactate} + \text{DPN}^+.$$

The increased availability of DPN^+ would then drive reaction [2] to the right.

[2] $$\text{Glyceraldehyde-3-phosphate} + \text{DPN}^+ \rightleftarrows \text{3-phosphoglycerate} + \text{DPN.H}.$$

The pyruvate used in reaction 1 would be regenerated in the reactions subsequent to reaction 2 and the elevated pyruvate concentration would be maintained except for the slow loss of pyruvate by dismutation[4] to acetate and lactate (10, 11).

The variability in previously observed rates of anaerobic glycolysis, mentioned in the Introduction, is readily understandable as being partly due to variations in the previous access of oxygen to the tissue. If the stimulatory effect of previous aerobiosis is due to restoration of high energy phosphate levels in the tissue, it is readily understandable why results of oxygen uptake measurements are far less variable since the concentration of ATP is less likely to be a limiting factor under aerobic conditions.

These studies suggest that the drastic irreversible changes in brain function in vivo which occur after short periods of cerebral anemia cannot be very well accounted for by irreversible loss of metabolic capacity, aerobic or anaerobic. Some slight irreversible damage to the systems concerned would occur, and to the capacity of the tissue to concentrate potassium (15). But the main effect of cerebral anemia seems more likely to be an effect of the loss of metabolic activity during the period of anemia on more delicate systems concerned with function. Our observations provide some reassurance that in vitro studies on energy metabolism by tissue slices are not likely to be vitiated by rapid irreversible damage to enzymes occurring during the time which elapses between the death of the animal, or excision of the tissue, and the commencement of measurements.

[3]The effects of previous aerobiosis and added pyruvate in increasing the rate of glycolysis over that found with untreated slices are usually approximately additive (15). This, however, is not very meaningful since an "untreated" slice is one which, in the course of ordinary manipulations, has undergone a variable degree of "previous aerobiosis" and is able to respond to pyruvate as well as to more complete "previous aerobiosis".

[4]The slowly developing inhibitory effect of added glutamate on brain glycolysis (16) may be due to the lowering of pyruvate concentration by transamination of pyruvate with the glutamate; the inhibitory effect is reversed by added pyruvate. On the other hand the inhibition might be due to a decrease in high energy phosphate concentration since McIlwain (17) found that the phosphocreatine content of respiring slices was decreased by the presence of added glutamate.

References

1. ELLIOTT, K. A. C. *In* Neurochemistry. *Edited by* K. A. C. Elliott, I. H. Page, and J. H. Quastel. Charles C. Thomas, Publisher, Springfield, Ill. 1955. p. 53.
2. DICKENS, F. and GREVILLE, G. D. Biochem. J. **27**, 1134 (1933).
3. MCILWAIN, H. Biochemistry of the central nervous system. J. & A. Churchill, Ltd., London. 1955.
4. CROSS, J. M.Sc. Thesis, McGill University. 1955.
5. ROSENTHAL, O. Biochem. Z. **233**, 62 (1931); **244**, 133 (1932).
6. ELLIOTT, K. A. C. and HENRY, M. J. Biol. Chem. **163**, 351 (1946); **163**, 361 (1946).
7. QUASTEL, J. H. and WHEATLEY, A. H. M. J. Biol. Chem. **119**, lxxx (1937).
8. MENDEL, B., BAUCH, M., and STRELITZ, F. Klin. Wochschr. **10**, 118 (1931).
9. BUMM, E., APPEL, H., and COUCIERO, P. Z. physiol. Chem., Hoppe-Seyler's, **220**, 186 (1933).
10. ELLIOTT, K. A. C., GREIG, M. E., and BENOY, M. P. Biochem. J. **31**, 1003 (1937).
11. KREBS, H. A. and JOHNSON, W. A. Biochem. J. **31**, 645 (1937).
12. MACFARLANE, M. G. and WEIL-MALHERBE, H. Biochem. J. **35**, 1 (1941).
13. PAPPIUS, H. M. and ELLIOTT, K. A. C. Can. J. Biochem. and Physiol. **34**, 1007 (1956).
14. PAPPIUS, H. M., ROSENFELD, M., JOHNSON, D. M., and ELLIOTT, K. A. C. Can. J. Biochem. and Physiol. **36**, 217 (1958).
15. PAPPIUS, H. M. and ELLIOTT, K. A. C. Can. J. Biochem. and Physiol. **34**, 1053 (1956).
16. WEIL-MALHERBE, H. Biochem. J. **32**, 2257 (1938).
17. MCILWAIN, H. Biochem. J. **52**, 289 (1952).

THE MECHANISM OF DRUG ACTION AT RECEPTOR SURFACES

PART I. INTRODUCTION. A GENERAL INTERPRETATION OF THE ADRENERGIC BLOCKING ACTIVITY OF β-HALOALKYLAMINES[1]

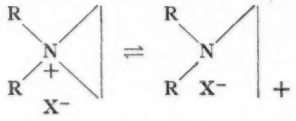
B. BELLEAU[2]

Abstract

An interpretation of the mechanism of action of "Dibenamine" is offered and the conclusion is reached that the excitatory receptors most probably include a carboxylate or phosphate anion as an active center. On the basis of a combination of isosteric and chemical reactivity principles, a precise correlation between structure and pharmacological activity of adrenergic blocking β-haloalkylamines is described. Structural similarities between sympathomimetic amines and adrenergic blockers are uncovered and applied to the interpretation of the activity of several hundred β-haloalkylamines. The crucial point of the discussion revolves about the concept of a *phenethylamine pattern* illustrated in Figs. 1 and 2 and it is demonstrated that this pattern applies generally to active adrenergic blocking β-haloalkylamines and that it allows predictions of activity.

Introduction

The chemical and biochemical importance of β-haloalkylamines has been established in most instances to rest upon their ability to form reactive ethylenimmonium ions under a wide variety of experimental conditions (8). The mechanism involved in the reactions of β-haloalkylamines has been studied in detail and the subject reviewed (33). For the purposes of the following discussion, it may be only necessary to recall the following pertinent facts: (1) Neutral hydrolysis of ethylenimmonium ions (here after abbreviated by EI-ion) leads to substitution of the most substituted carbon atom (19) in agreement with the partial carbonium ion character of the ring carbons of the EI-ion (14, 33). It would appear that the best structure for EI-ions should



(A) (B)

be that of an intimate ion-pair (B) in equilibrium with (A). The three-membered EI-ion being a strained ring (33), it is obvious that a wide variety of nucleophilic groups will tend to associate with the ring carbons to form aggregates of lower free energy content and lead to ring opening whenever possible. (2) The rate of formation as well as the stability of EI-ions should increase as the basicity of the amino group increases, a presumption confirmed by Cohen Artsdalen, and Harris (4).

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(3) Proteins are known to react with EI-ions, and it has been recently and conclusively demonstrated by Goodlad (9) that the reaction occurs mostly at the site of carboxylate anions leading to labile esters. The sensitivity of such esters to hydrolysis is in keeping with the previous observation of Blackburn, Carter, and Phillips (2) on the remarkable lability of methyl esters of wool protein at alkaline pH. It has also been shown by Davis and Ross (5) that esters of β-dialkylaminoethanol are quite sensitive to alkaline hydrolysis, a fact which may best be explained on the basis of neighboring group participation (compare with ref. 5). Strong presumptive evidence that EI-ions attack mainly the carboxyl groups of proteins had previously been adduced by Ross (31) and several other groups of investigators (17). It has also been demonstrated (7) that the phosphate anions of the nucleotides are readily esterified by EI-ions. The biological effects of mustard compounds can therefore best be interpreted in terms of a temporary blockade of carboxylate anions followed by regeneration through hydrolysis, a presumption already discussed by others (5, 30).

Alkylation of other nucleophilic groups such as amino groups, thiols, sulphides, or hydroxyl groups by EI-ions also occurs to some extent but usually in an irreversible manner (6). Thus, in vivo regeneration of this type of alkylated groups should be much more sluggish and may necessitate total *de novo* resynthesis of the affected protein in order to recover these functional groups.

Adrenergic Blocking Agents of the "Dibenamine" Series

In 1945, Nickerson and Goodman (25) first reported the powerful adrenergic blocking activity of N,N-dibenzyl-β-chloroethylamine ($(C_6H_5CH_2)_2NCH_2CH_2Cl$, "Dibenamine") and the circumstances that led to this key discovery have been most interestingly related by Ullyot and Kerwin (34). Since the discovery of this drug, several hundred β-haloalkylamines have been synthesized and evaluated pharmacologically and an excellent comprehensive survey of the field has been made recently by Ullyot and Kerwin (34). Some time ago, Nickerson summarized his views on the pharmacology of adrenergic blockade (23) and recently reinterpreted the mechanism of action of adrenergic blocking β-haloalkylamines (24). His original opinion that the pharmacologically active species is inherent to the EI-ion easily derivable from "Dibenamine" in buffered solutions (23) was subjected to some refinements and the term "non-equilibrium antagonism" applied to describe the pharmacological properties of β-haloalkylamines (24). Chapman and James (3) also reached similar conclusions concerning the chemical nature of the pharmacologically active species. Although no adequate rationalization is yet available of the selective activity of "Dibenamine" and related compounds, efforts in that direction have led to the following salient conclusions: (1) EI-ions constitute the actually active species (3, 15, 23, 26). (2) The type of blockade produced by "Dibenamine" (26) and its congeners is of the non-equilibrium type (24) and is overcome only by time (26). It follows therefore that the effects of

β-haloalkylamines can be ascribed to an alkylation reaction by the EI-ion of a receptor substance (15, 23, 24). (3) The prolonged duration of blockade is ascribable to a slow enzymic removal of the drug from the receptor, and variations in the duration of blockade produced by various β-haloalkylamines can be explained by variations in the rate of splitting of the bond linking the drug to the receptor (24). However, the slow release from fat depots of accumulated drug may be of some significance in the whole animal (1, 34). (4) Two phases are discernible in the establishment of adrenergic blockade (24): (*a*) the EI-ion is attracted by the receptor and retained by weak forces; (*b*) the EI-ion then reacts chemically with the receptor. (5) According to Nickerson (24), the nature of the entity undergoing alkylation is best defined as a thiol group although this idea is difficult to reconcile with a number of facts as will become evident later. (6) In some cases, there exists a relation between chemical reactivity of the β-haloalkylamines and intravenous potency of the blocking agents (34) provided only that the effect of the nature of the halogen is considered. However, amongst the various structures no correlation between chemical reactivity and pharmacological potency is discernible (34). (7) Although a vague suggestion of hyperconjugation and resonance effects acting as possible factors controlling biological activity has been offered (23), but pertinently criticized elsewhere (3), it is quite apparent that, quoting Nickerson (23)" . . . the question why certain chemical structures and not others produce effective blockade remains unanswered".

Nickerson's recent interpretation (24) of the nature of the adrenergic blockade produced by "Dibenamine" EI-ion requires the postulate that the rate of splitting of the bond linking the drug to the receptor varies for different drugs in order that the observed variations in the duration of blockade can be explained. Although this explanation appears most reasonable, it does not seem essential to postulate that such splitting is enzymatically catalyzed since it is only necessary to invoke purely chemical factors as will presently become evident. If the receptors have the properties of a protein it is clear that exposure to an alkylating agent can lead to introduction of alkyl substituents on amino, thiol, hydroxyl, and carboxyl groups. Once alkylated, the first three groups would not be expected to be easily regenerated, as already pointed out above. Moreover, the weight of recent evidence summarized above (9, 17, 31) clearly indicates that carboxylate and phosphate anions are preferred targets for EI-ions and lead to hydrolyzable esters which can conceivably undergo slow (prolonged activity) or fast (short duration) hydrolysis depending on the degree of anchimeric assistance by adjacent basic groups. In turn, the degree of participation of the latter groups will depend upon the degree of interaction or shielding produced by the substituents on the drug. In general, therefore, it may be expected that small substituents on the β-haloalkylamine should lead to quicker regeneration of the original carboxyl group thus leading to rather short duration of blockade. This appears to be the case although the chemical nature of the substituents should be taken into account before any generalizations can be drawn. According to this reasoning, the tentative but logical

conclusion may be reached that the excitatory adrenotropic receptors include amongst their binding sites for sympathomimetic agents, *a carboxylate or phosphate anion*, which can be masked by reaction with an appropriate EI-ion.

The Mode of Action of "Dibenamine"

Nickerson's statement (23) that ". . . little is gained by attempts to squeeze blocking molecules into the phenethylamine mold" is particularly true if the classical concepts of isosterism are taken at their face value and not accurately specified in terms of chemical and conformational principles. With due regard to the probable steric requirements of the "mold", it appeared of fundamental interest to attempt to correlate biological activity with structure by a judicious combination of isosteric and chemical principles. Since it appears well established that adrenergic blocking β-haloalkylamines selectively abolish the excitatory responses normally produced by a variety of sympathomimetic amines (28), it is clear that they must interact with the same excitatory receptors and that a common basic structural pattern must relate them. Moreover, the structural and chemical requirements for vasopressor activity of sympathomimetic amines should grossly reflect the steric and chemical requirements of the excitatory receptor surface. Thus, it can be safely asserted that strong vasopressor activity is usually associated with the presence in a molecule of a 2-phenylethylamine entity although the fact that certain aliphatic amines such as 2-aminoheptane are vasopressor makes the presence of a benzene ring only a relative requirement for such activity. Nevertheless, it is quite reasonable to assume that two major binding sites occur on the receptor surfaces, respectively involved with the amine function in its protonated form (site A, Fig. 1) and the aromatic moiety (site B). It can further be assumed that it is the interaction of a pressor drug with site A which triggers an excitatory response and that site B contributes mainly to efficacious binding of the drug, a role that may become an essential one if affinity of an onium group for site A is insufficient to produce effective binding on the receptor. The nature of site A can be defined as a *carboxylate or phosphate anion*, a most logical binding center for an onium group and can be considered identical with the carboxylate or phosphate anion already suggested to be present on the receptor (see above). Obviously, other active centers also occur on the excitatory receptor surface and undoubtedly contribute to a still more efficacious binding of agonists. These binding centers (sites P and M) are respectively concerned with the two phenolic hydroxyl groups of epinephrine and norepinephrine. A binding center (not shown) for the alcoholic hydroxyl group of epinephrine must also be present but its role has been shown by Lands (21) to be essentially associated with inhibitory responses. The most important feature is the distance separating sites A and B and for the purposes of the present discussion, reference will be made to the "*phenethylamine pattern*" in order to indicate that *regardless of the nature of the functional groups, a given molecule will be considered potentially active either as an agonist or antagonist if it includes a group capable of interacting with site A and if one of the probable conformations allows this group to be located*

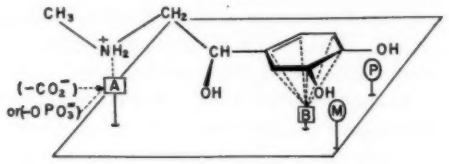


FIG. 1

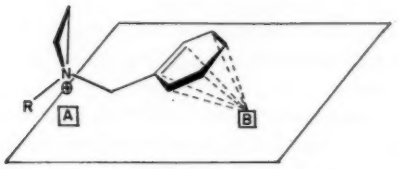


FIG. 2

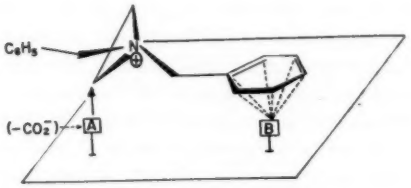


FIG. 3

at approximately three interatomic distances away from a ring possessing aromatic character. Thus, normally, a four-point contact of agonist or antagonist molecules with the excitatory receptor will be involved and rigidly corresponds to site A (—COO^- or PO_3^-), which is essential; site B, which is necessary under most circumstances (see above); sites P and M, which are optional but capable of either favoring adsorption or creating interference with adsorption at B, depending on the nature and size of the substituents on the benzene ring. From these definitions, a clear-cut relationship between "Dibenamine" EI-ion and the assembled excitatory receptor emerges and is shown in Fig. 3. The most striking and superficially objectionable feature introduced in Fig. 3 is the fact that a carbon atom of the EI-ion is equated to the ammonium group of a phenethylamine and that it would seem more reasonable to assume binding through the quaternary nitrogen (Fig. 2). Indeed, this may very well be the case but only as a primary step in the establishment of blockade and might conceivably correspond to the first phase, which has been elegantly shown by Nickerson (24) to involve true reversible antagonism. Because the ring carbons of EI-ions do possess *carbonium ion character*, as pointed out above, they should have affinity for the anion of site A, and slow rearrangement from Fig. 2 to the conformation shown in Fig. 3 should be especially favored by the close adherence of the molecule to the "phenethylamine pattern". Alkylation of A should simultaneously ensue and would correspond to Nickerson's second phase (24) of non-equilibrium antagonism. This interpretation also agrees

remarkably well with the demonstration that EI-ions constitute the exclusive active species (3, 15, 26). Next, once alkylation of A has occurred, a more or less labile ester is produced and the "phenethylamine pattern" necessary for interaction with the receptor is destroyed, thus leaving it to other factors to affect the rate of hydrolysis of the ester linkage. As already pointed out above, one such factor might be vicinal basic groups which, according to the degree of overcrowding or shielding produced by the substituents, can more or less assist hydrolysis of the ester function. For the first time, therefore, the physiological activity of "Dibenamine" and its congeners becomes understandable in terms of chemical and isosteric principles involving the excitatory receptors as the target.

A more refined examination of the preceding interpretation reveals that the transition from Fig. 2 to Fig. 3 may require the stimulus of a sizeable attraction of the benzene ring by site B leading, by an appropriate sliding of the molecule, to the "phenethylamine pattern" which in turn is conducive to alkylation of A. Without this pull exerted upon the ring, it is probable that the fractional positive charge of the ring carbons of the EI-ion would not alone be conducive to the requisite sliding leading to alkylation of A because of the favorable competition by the quaternary nitrogen. As a consequence the molecule would at best produce only reversible antagonism and suffer desorption without ever alkylating site A. It seems clear therefore that the presence of a suitably placed aromatic ring in β-haloalkylamines should constitute an absolute requirement for adrenergic blocking activity in contrast to pressor amines for which this requirement is only relative owing to the highly polar character of ammonium ions leading per se to powerful electrostatic interaction with site A. An explanation is thus provided for the inactivity of purely aliphatic or alicyclic β-haloalkylamines (34).

The Effect of Substituents on the Aromatic Ring

It would be extremely presumptuous to expect quantitative predictions of the effect of substituents on adrenergic blocking activity because of the numerous chemical and biochemical variables as yet unknown that must come into play. Such factors as chemical reactivity of the β-haloalkylamine and its corresponding EI-ion as influenced by substituents are not known and the same is true of the half-life of the drug in the animal, its mode of degradation, and its capacity to penetrate tissues and reach the receptors. Moreover, the pharmacological evaluation itself is subject to a number of experimental variables so that only the range of activity has real significance. Quantitative knowledge of the effect of substituents on the electron density and distribution in the aromatic ring would also be required and can only be grossly approximated. It is clear therefore that on the basis of the presently available knowledge, predictions of activity can at best be of a qualitative nature, but this would nevertheless mark an important step forward if it could be achieved.

From correlation studies alone, it is not possible to arrive at a reasonable interpretation of the effect of ring substituents on activity, but, if due consideration is paid to the configuration and chemical requirements of the receptor

surface, which remains constant, it becomes possible to foresee some general trends in the effect of such substituents on activity. From the fact that the catechol nucleus is the normal substrate for the receptor (Fig. 1) and that its presence leads to intense pressor activity in agonist molecules, it can be deduced that site B of the receptor has increased capacity to complex with an aromatic ring if the basicity of the latter (or electron density) is increased in proportion. The strong +E effect of the two hydroxyl groups of catechol accounts for the higher electron density of the ring and in the field of β-haloalkylamines the general trend should be therefore that *substituents with a +E or +I effect should tend to preserve activity*, assuming all other factors to be equal. On the other hand, if a combination of substituents have a very marked −I effect, thus considerably deactivating the ring, it can be expected that it may lose its capacity to complex with site B and that adrenergic blocking activity may disappear. It is immediately apparent that the nature and position of the substituents should impose certain limitations to the preceding generalization in view of the fact that the requirements of sites M and P may not allow complexing at B if repulsive forces are created at these two points. Thus, (1) in connection with the position of a group, it is clear that substituents on the 2-position of the benzyl group of "Dibenamine" congeners, are much less likely to interfere with sites M and P and this position should therefore be the best choice for a substituent if blocking activity is to be preserved. The same is true of a group in the 3-position which can readily avoid the M site through a 180° rotation of the ring (Fig. 4). In contrast, the 4-position should greatly

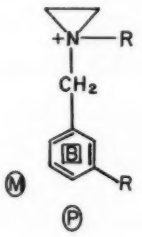


FIG. 4

restrict the choice of the substituent since rotation of the ring serves no purpose in that case. Hence, a decreasing order in the importance of the limitations imposed by the position of the groups should prevail and corresponds to: para ≫ meta ⩾ ortho. At first approximation, therefore, only substituents in the 4-position are likely to interfere drastically with adrenergic blocking activity. (2) Concerning the nature of these substituents, besides the idea that they should have a +E or +I effect in preference to a very strong −I effect, the choice should be made so that departure from the size or polar characteristics of a hydroxyl group is kept at a minimum. A specification of the types of groups complying with these restrictions is difficult to arrive at,

since the nature and shape of the P center of the receptor is unknown. However, from the known effect on pressor activity of certain groups in the 4-position of agonist amines which are known to interact with the same excitatory receptors as has already been demonstrated, some indication relating to the selectivity of site P can be obtained, although it does not necessarily follow that similar substituents in agonists and antagonists should affect activity in the same direction. Nevertheless, on that basis, it would appear that the type of substituent other than a hydroxyl group in the 4-position of "Dibenamine" that is likely to preserve adrenergic blocking activity should be as small as possible, not bigger than (OCH_3) for an ether, (CH_3) for an alkyl, and F for the halogens. It is conceivable, however, that the addition of another +E substituent on the ring could, by increasing the basic character of the

TABLE I*

Compound	Character of the substituent	Compatibility with P†	Predicted activity	Observed range
$C_6H_5CH_2N(CH_2CH_2Cl)(C_2H_5)$			A	A
Substitution in phenyl ring by:				
(1) 2-CH_3	+I		A	A
(2) 2-OCH_3	+E≫−I		A	A
(3) 4-OCH_3	+E≫−I	C (11)	A	Inact. (see text)
(4) 3-CF_3	−I		A	A
(5) 3-Cl	−I>+E		A	A
(6) 4-C_2H_5	+I	X (13)	Inact.	Inact.
(7) 4-Cl	−I>+E	Doubtful	Inact.	Slight A
(8) 3,4-$(OCH_3)_2$	+E, +E	±C (10)	Slight A	Slight A
(9) 3,4-(OCH_2O)	+E, +E	C (12)	A	Slight A
$(C_6H_5CH_2)_2NCH_2CH_2Cl$			A	A
Substitution in one ring by:				
(10) 2-OCH_3	+E		A	A
(11) 3-CF_3	−I		A	Slight A
(12) 3-I	−I>+E		A	A
(13) 4-CH_3	+I	C (22)	A	A
(14) 4-OCH_3	+E≫−1	C (11)	A	A
(15) 3,4-$(OCH_3)_2$	+E, +E	±C (10)	A‡	A
(16) 3,4-(OCH_2O)	+E, +E	C (12)	A	Slight A
Substitution in both benzyls by:				
(17) 2-CH_3	+I		A	A
(18) 3-CH_3	+I		A	A
(19) 3-CF_3	−I		A	Slight A
(20) 3-Cl	−I>+E		A	Slight A
(21) 4-CH_3	+I	C (22)	A	A
(22) 4-C_2H_5	+I	X (13)	Inact.	Inact.
(23) 4-OCH_3	+E≫−1	C (11)	A	A
(24) 2,4-Cl_2	−I, −I	(Ring strongly deactivated)	Inact.	Inact.
(25) 3,4-Cl_2	−I, −I	"	Inact.	Inact.

*The observed data were taken from Ullyot and Kerwin's review (34). Significant activity is designated by A and, unless otherwise noted, does not refer to the degree of activity.

†This refers to the absolute compatibility of a 4-substituent with site P and is based on the positive or negative effect of the substituent on the pressor activity of sympathomimetic amines. The letter C means compatible, X incompatible, and the number in parenthesis refers to the literature where the data were obtained.

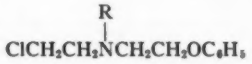
‡In case of incompatibility at P, the unsubstituted benzyl is considered to interact with site B (see text, Figs. 1, 3).

ring, outweigh to some degree an unfavorable interaction of a 4-substituent with site P and help preserve adrenergic blocking activity. The reverse effect should be observed with an additional −I substituent. No such specification of the size or nature of substituents in the 2- and 3-positions is necessary because as pointed out above, interaction with the M and P centers is avoided. (3) It is clear that "Dibenamine" relatives carrying substituents in only one of the rings should approximate "Dibenamine" itself in potency because of the possibility for interaction through the unsubstituted ring with the receptor, thus eliminating any unfavorable interaction of the substituents with the receptor. (4) It should also be noted that the weaker the basic character of a nucleus, the stronger should the effect of substituents be. Hence, inherently strongly basic aromatic nuclei should lead to such powerful complexing ability with site B that repulsive forces from sites P and M may be effectively outweighed.

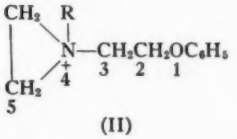
In Table I, predictions of activity as deduced from the above premises are compared with the observed values in a series of "Dibenamine" related compounds. Whenever necessary and possible, reference is given to known effects of substituents on the pressor activity of sympathomimetic amines and these effects are used as a basis in the deduction of absolute compatibilities with site P. It is striking that qualitative agreement with observation is almost complete, thus pointing to a remarkable degree of validity in the selected premises. Exceptions are compounds 3, 11, 19, and 20, of which only compound 3 gives radical disagreement with prediction. It would seem that the latter compound should be pharmacologically reevaluated before any explanation is sought for this striking discrepancy.

The Phenoxyethylamine Series

In apparent conflict with the above interpretation of the adrenergic blocking activity of Dibenamine and its congeners, it was discovered by Nickerson and Gump in 1949 (27) that a series of N-(2-phenoxyethyl)-N-alkyl-β-chloroethylamines (I) possess adrenergic blocking activity similar in nature to Dibenamine. It was later shown by Harvey and Nickerson (15) that here again, the corresponding EI-ion (II) constitutes the actually active species. It is clear that (II) deviates from the "phenethylamine pattern" required for activity and it does not seem possible to accommodate (II) on the receptor because of the five interatomic distances separating a ring carbon of the EI-ion and the aromatic ring. Yet, several derivatives of (II) are highly effective blocking



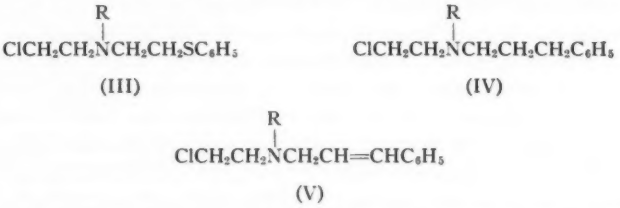
(I)



(II)

agents and it is noteworthy that some phenylthioethyl analogs (III) are also active. Significantly, however, hydrocinnamyl isosteres (IV) are devoid of

activity whereas cinnamyl derivatives (V) are quite effective blockers. No explanation is immediately apparent for these remarkable observations and



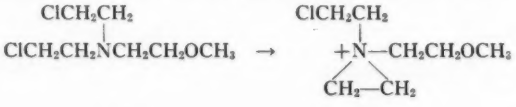
(III) (IV)

(V)

it is obvious that hyperconjugation effects (23) are of minor significance. Again, the effectiveness of these compounds must be related a priori to a particular fit on the epinephrine receptor mold and an important clue to this problem is supplied by the biological inactivity of the hydrocinnamyl derivatives (IV). Thus, it is apparent that the ethereal oxygen of (I) must play a crucial role in the appearance of activity and it becomes pertinent to examine first the possibility that the chemical reactivity of the β-haloalkylamine moiety is affected by the presence of the ethereal oxygen. It has been shown for instance that anchimeric assistance of bromide ion release by a vicinal alkoxyl group occurs with 4-bromobutyl ethyl ether (VI) and is reflected in a large increase in the rate of bromide liberation when comparison is made with lower or higher homologs (29). Obviously, the extremely facile formation of the

(VI) (VII)

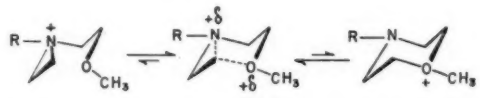
five-membered transition state (VII) offers the best explanation for the observed effect (29). It is known that given the opportunity, the tetrahydrofuran ring can form with extreme ease (16). Of more immediate consequence yet to the case under discussion is the observation of Cohen, Artsdalen, and Harris (4) that 2-methoxyethyl-N-bis-(2-chloroethyl) amine (VIII) cyclizes to its corresponding EI-ion (IX) at a rate 10 times greater than simple aliphatic analogs. However, no explanation was offered for this observation which can be ascribed to an effect of the oxygen atom analogous to the one operating



(VIII) (IX)

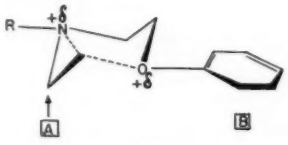
in the case of (VII). The ring carbons of the EI-ion (IX) having cationic character (*vide supra*), it is clear that the vicinal nucleophilic oxygen should anchimerically interact with these carbons in such a way as to dampen chloride

ion return and consequently increase the rate of liberation of the latter. It is apparent therefore that a transition state (X) of lower free energy content than (IX) must form since, in the extreme, the tautomeric strainless six-membered structure (XI) allows the release of strain from the EI-ion (IX).



(IX) (X) (XI)

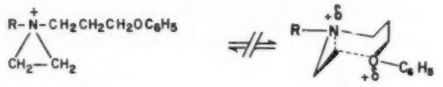
The conclusion is thus permissible that an oxygen atom (or any other heteroatom) situated as in (IX) with respect to the three-membered EI-ion (a situation comparable to the case of 4-bromobutyl ethyl ether discussed above) should lead to a transition state such as (X) which has fixed geometry and that the proportion of molecules as well as the stability of this hybrid structure will depend upon the nucleophilic power of the heteroatom.[3] Application of these principles to the case of N-(β-phenoxyethyl)-N-alkyl-β-chloroethylamines (I) (II) leads to the formulation of a similar transition state (XII), the existence of which is supported by the fact that Dibenzyline cyclizes at a rate 5 to 10 times greater than Dibenamine (15, 34).[3]



(XII)

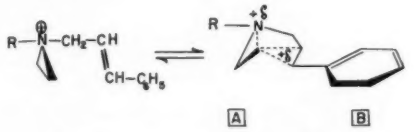
The geometry of (XII) is such that the phenethylamine pattern requiring that a cationic carbon be present at the equivalent of three interatomic distances from an aromatic ring is strictly respected and consequently conducive to a perfect fit on the theoretical epinephrine receptors. In complete agreement with this interpretation, phenoxyethylamine derivatives (I) exhibit a high degree of adrenergic blocking activity as mentioned above. Now, if this interpretation is correct, it would be expected that hydrocinnamyl isosteres (IV) would be devoid of activity since no transition state analogous to (XII) can possibly form. In fact, compounds of that series (IV) are inactive. On the other hand, replacement of the oxygen of (XII) by sulphur should produce active derivatives in view of the high nucleophilicity of sulphur which allows the formation of a transition state isosteric with (XII). Indeed, this is found to be the case and compounds of that series (III) are active. However, the homologous 3-phenoxypropylamine derivatives (XIII) should be devoid of activity, because little participation of the oxygen in establishing a fixed conformation (XIV) would be expected on the basis of the feeble assistance by

[3]Results to be described in a forthcoming paper as Part III of this series agree with this interpretation.



(XIII) (XIV)

oxygen of bromide ion release in a 5-bromoether (29) and also because the entropy demand in the formation of the seven-membered ring (XIV) is too high. In agreement with this reasoning compounds of the class of (XIII) are inactive. The case of cinnamyl derivatives (V) requires special attention since the high degree of adrenergic blocking activity may seem surprising if comparison is made with the inactive hydrocinnamyl analogs (IV). Nevertheless, examination of the corresponding EI-ion (XV) reveals that because of the trans-configuration of the cinnamyl moiety and of rotational isomerism, a statistically significant proportion of the molecules can adopt configuration (XV) which obeys the phenethylamine pattern rule for high activity. The fraction of the total number of configurations existing as the rotational isomer (XV) can be significantly increased by interaction of the pi-electrons of the double bond with a cationic carbon of the EI-ion as shown in formula (XVI). However, the magnitude of this latter interaction may not be so great as to allow the detection of species (XVI) by the usual methods of chemical kinetics. However, neighboring double bond participation in the solvolysis of certain halides has been well demonstrated (32) and offers some support for the formulation of (XVI). Again, no analogous situation is possible with the parent hydrocinnamyl derivatives.



(XV) (XVI)

The effect of substituents on the aromatic ring of adrenergic blocking N-(2-phenoxyethyl)-N-alkyl-β-chloroethylamines (I) (Table II) can be anticipated to alter activity in two ways: (1) as in the Dibenamine series, +E or +I substituents preferably in the 2-position (the 3-position being second in choice) should tend to improve activity because of reasons already discussed above; (2) the same substituents will also increase nucleophilicity of the ethereal oxygen atom and consequently facilitate formation of the requisite transition state (XII) and again lead to improved blocking ability. Hence, activity of phenoxyethylamine derivatives (I) should be more deeply affected by the presence or absence of suitable substituents on the ring than Dibenamine congeners because of the superimposition of this additional factor. This is borne out by the observation (23, 34) that the presence of a 2-methyl group in the ring of the phenoxyethyl moiety of (I) produces a large increase in adrenergic blocking activity as compared to the effect of a 2-methyl group in the Dibenamine series. In fact, a 2-methyl substituent in this latter series has an

TABLE II

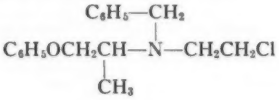
Compound	Adrenergic blocking activity*	
	Observed range	Predicted range
(26) $C_6H_5OCH_2CH_2N(C_2H_5)CH_2CH_2Cl$	A	A
Substitution on the phenyl ring by:		
(27) 2-CH_3,2-C_2H_5	A	A
(28) 2-OCH_3	A	A
(29) 2-C_6H_5	A	A
(30) 2-$CH_2C_6H_5$	A	A
(31) 3-CH_3	Inact.	A
(32) 3-CF_3	Inact.	Inact.
(33) 3-C_6H_5	A	A
(34) 4-CH_3	A	A
(35) 2-OCH_3,4-$CH_2CH{=}CH_2$	Inact.	Inact.
(36) $C_6H_4(CH_3(2))SCH_2CH_2N(C_2H_5)CH_2CH_2Cl$	A	A

*See footnote, Table I, for the E or I effect of the substituents and the compatibility of a 4-substituent with site P. A phenyl group is taken as a +E substituent since biphenyl is more basic than toluene (see below in connection with ref. 20).

opposite effect which may be ascribable to steric interference with the chemical reactivity of the β-haloalkylamine moiety. Evidence on that point as well as on the stabilizing influence of a 2-methyl group on transition state (XII) in the phenoxyethylamine series is forthcoming and will be reported separately. As can be judged from Table II, predictions of activity on the above grounds agree in most cases with experimental observation and lend strong support to the concepts exposed thus far. An exception is compound 31, which is reported to be inactive even though there is no apparent reason for this. This contrasts markedly with the effect of a 3-methyl group in the Dibenamine series and suggests that the relationship of (XII) with the postulated receptor is subject to rather subtle steric requirements that will require closer examination. The often erratic effects of ring-substitutions is best illustrated by the fact that disubstituted phenoxyethylamines appear to be uniformly inactive irrespective of the nature and position of the substituents. These observations cannot be readily brought into the pattern of the present interpretation at the present time, but can be expected to yield valuable and perhaps fundamental information if subjected to refined physicochemical investigation as well as to conformational analysis of transition state (XII).

All other compounds of the phenoxyethylamine series carrying a benzyl group on the nitrogen (such as "Dibenzyline" (XVII), can be considered as "Dibenamine" relatives but with the additional feature that the chemical reactivity of the β-haloalkylamine moiety is influenced by the oxygen of the phenoxyethyl portion. Thus, (XVII) can interact with the receptor as a

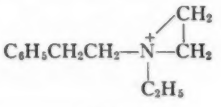
"Dibenamine" relative or as a phenoxyethylamine derivative through transition state (XII). A discussion of the effect of substituents on the rings of "Dibenzyline" and its congeners would add little to the fundamental points of the present discussion and is deleted purposedly.



(XVII)

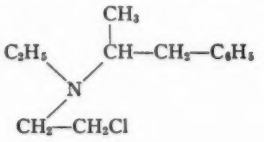
The Phenethylamine Series

On the basis of the postulated requirements of the adrenotropic receptors, N-(β-haloalkyl)-N-alkyl-phenethylamines in the form of their corresponding EI-ions (XVIII) should not be active adrenergic blocking agents in view of the deviation from the requisite phenethylamine pattern. In fact, such derivatives are almost inactive (23, 34) in agreement with an unfavorable position of a cationic carbon with respect to the aromatic ring. There is one exception



(XVIII)

however, and this is N-(β-chloroethyl)-N-ethyl-phenylisopropylamine (XIX), which produces adrenergic blockade approaching "Dibenamine" in potency (34). Even though this observation cannot be reconciled with the structure–activity rules presented above, it is possible on different grounds to account for the activity of (XIX). It is noteworthy that compounds related to (XVIII) do exhibit activity albeit of a very low order, and it is apparent that if a very high concentration of reactive EI-ions, but poorly fitting the receptors, can



(XIX)

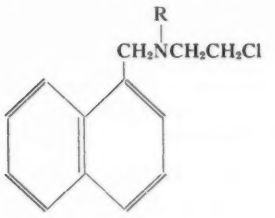
be maintained, the yield of alkylated receptor will be increased in proportion. Now, the fact that (XIX) gives rise in buffered solution to a very high concentration of EI-ion (34) exceeding by far the concentration obtainable with "Dibenamine" strongly suggests that this increased concentration is sufficient to raise the expectable low activity of (XVIII) to the higher level of (XIX). Cogent evidence in support of this interpretation is adduced by the fact that N-(β-bromoethyl)-dibenzylamine, which cyclizes to the corresponding EI-ion at rate 15 times greater than "Dibenamine", is correspondingly 5 to 10 times

more active than the latter (34). Clearly, then, concentration gradient effects at the level of the receptor can be of importance from the quantitative point of view although not from the qualitative one.

Polynuclear β-Haloalkylamines

A discussion of the adrenergic blocking activity of β-haloalkylamines carrying condensed polynuclear aromatic systems requires specific knowledge of the relative basicity of the most common aromatic nuclei in view of the premises ascribing affinity for site B of the receptor to the basic character of the aromatic moieties. Valuable information on that point is available from the work of Kortüm and Vogel (20), who studied the dissociation of iodine complexes of common aromatic systems and could deduce the following order of increasing basicity: chlorobenzene < toluene < naphthalene < biphenyl < fluorene < acenaphthene < 2,6-dimethylnaphthalene < phenanthrene < 2,3-dimethylnaphthalene <- - - - - <anthracene. According to this relative basicity scale, it would be expected that by substituting the benzyl group of N-alkyl-N-(β-chloroethyl)-benzylamines for any one of those ring systems situated above toluene in the scale, an order of adrenergic blocking activity higher than "Dibenamine" should result because of the increased affinity of the new ring for site B. It is remarkable indeed that predictions of activity based on these presumptions are in perfect agreement with experimental observation (34), thus adding considerable weight to the speculations exposed thus far. One striking exception, however, is found in β-naphthylmethylamine derivatives (XXVI) and is discussed below. Examples (34) are the α-naphthylmethylamine derivatives (XX) which exhibit 5 to 10 times the activity of "Dibenamine". Some biphenyloxyethylamine derivatives (XXI) exhibit even higher activity; some 9-fluorenylamine compounds (XXII) are also much more active than Dibenamine; acenaphthenyl derivatives (XXIII) are highly active whereas the 9-phenanthrylmethylamine compound (XXIV) is twice as active as "Dibenamine". Finally, the recently described (18) anthracene derivative (XXV) is much more potent than "Dibenamine".

As in the case of Dibenamine, the presence of substituents on these aromatic nuclei should influence activity to some degree but very few studies have been carried out in that direction. The prediction is permissible, however, that those positions on the aromatic rings which are equivalent to the 4-position of "Dibenamine" or which overlap with site P of the receptor should impose some restrictions as to the choice of the substituent and a tendency toward decreased activity may be expected if there is marked departure from hydroxyl or methoxyl groups. On the other hand, *and in contrast to the "Dibenamine" series*, as has been already pointed out above, it can be anticipated that as the basicity of the aromatic systems increases, substituent effects should decrease in importance because with the more basic ring systems, *complexing ability with B may become so great as to outweigh adverse repulsive forces from other sites*. Generally speaking, therefore, simple substituents on the highly basic aromatic systems may influence adrenergic blocking activity less significantly.



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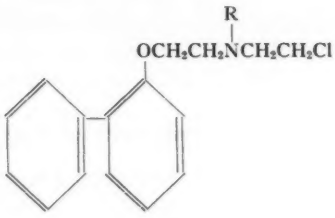
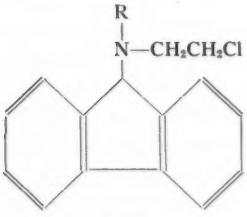


(XXI)



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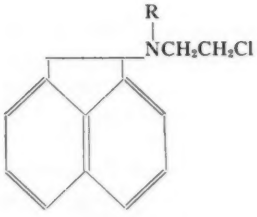

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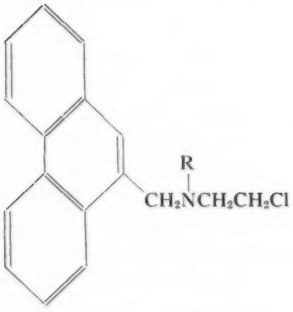
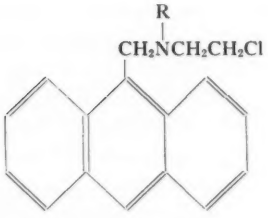
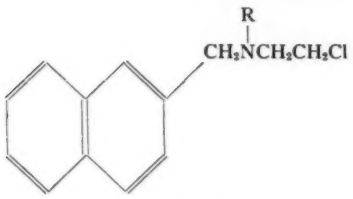


(XXIV)



(XXV)

A disturbing exception to the above predictions of activity as based on the basicity scale advocated by Kortüm and Vogel is found in the N-(β-naphthylmethyl)-N-alkyl-β-chloroethylamines (XXVI) which are devoid of activity (34) in spite of the expectable adequate affinity of the naphthalene nucleus for site B of the receptor. It has further been shown (3) that the absence of adrenergic blocking activity in (XXVI) cannot be ascribed to altered chemical reactivity of the β-haloalkylamine moiety. However, these puzzling observations can be most readily explained if the β-naphthylmethyl group of (XXVI) is looked upon as a benzyl group carrying an unsuitable substituent in the 4-position, a situation already shown (*vide supra*) to destroy activity in the



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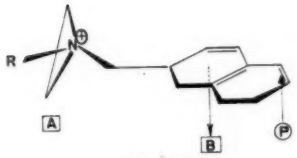
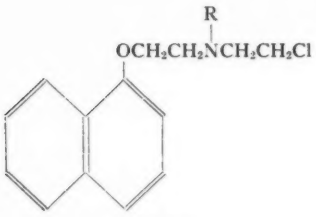


(XXVII)

"Dibenamine" series. As can be seen in (XXVII), repulsion at P can outweigh affinity at B and bring about desorption before alkylation of A can take place. Clearly, such is not the case with α-naphthylmethylamine derivatives (XX) since little interference with P can occur. Cogent evidence favoring this view lies in the fact that certain alkyl groups such as ethyl in the 4-position of "Dibenamine" similarly abolish activity (see above). In keeping with this interpretation of the inactivity of (XXVI), it would be expected that if affinity for B is further increased, it may become possible to outweigh repulsive forces from P and cause adrenergic blocking activity to reappear. Indeed, this appears to be the case since the 9-phenanthrylmethylamine derivative (XXIV), which includes a β-naphthylmethyl entity as a portion of its structure, is an active adrenergic blocker in agreement with the greater basicity of phenanthrene as compared to naphthalene (20). This would tend to confirm the view already expressed above that, with the inherently highly basic aromatic systems, complexing ability with site B may become so great that repulsion at P loses significance. It would be of interest in this connection to test β-naphthylmethylamine derivatives carrying two methyl groups on the ring, since, according to the above basicity scale, 2,3-dimethylnaphthalene is even more basic than phenanthrene. Investigations along these lines are under way and will be reported later.

By analogy with the activity exhibited by phenoxyethylamine derivatives (I), it can be expected that α-naphthoxyethylamine compounds (XXVIII) should also be active, provided the ethereal oxygen is sufficiently nucleophilic to allow formation of a transition state analogous to (XII). In fact, such derivatives (XXVIII) possess adrenergic blocking activity (34) in the range of simple phenoxyethylamine compounds. On the other hand, β-naphthoxyethylamine derivatives (XXIX) are devoid of activity for reasons already discussed in the case of β-naphthylmethylamine relatives (XXVI, XXVII).



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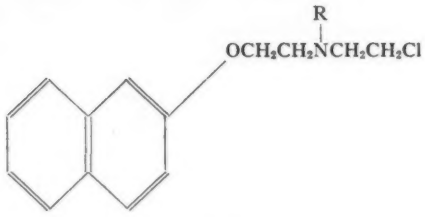
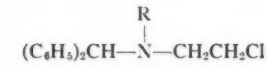


(XXIX)

Other classes of β-haloalkylamines leading to good fits on the theoretical receptor (Fig. 3) and which do not require special comment include benzhydryl (XXX) and indanyl derivatives (XXXI).



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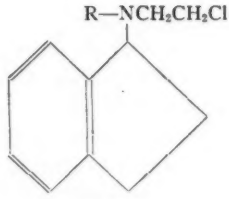
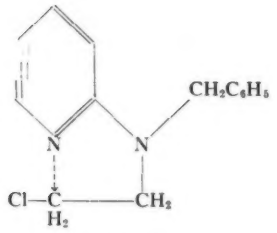


(XXXI)

Adrenergic blocking haloalkylamines carrying an heterocyclic substituent on the nitrogen do not offer any serious difficulty as far as interpretation of activity is concerned if only simple furfuryl or thenyl derivatives are involved. The truly aromatic character of these heterocycles suffices to satisfy affinity requirements for site B. Moreover, all those compounds carrying a benzyl substituent on the nitrogen fall within the class of "Dibenamine" relatives and consequently should approximate the latter series in activity. However, a few anomalies are apparent, such as the inactivity of N-(2-pyridyl) (XXXII), N-(4-quinolinemethyl) (XXXIII), and N-(4-imidazolemethyl)-N-(β-haloalkyl)-benzylamine (XXXIV). In contrast, N-(2-pyridylethyl)-N-benzyl-β-chloroethylamine (XXXV) is an active adrenergic blocking agent.

As shown in (XXXII) and (XXXIV) prevention of EI-ion formation is very likely to occur because of the vicinal basic nitrogen of the heterocyclic rings. However, (XXXV) should form an EI-ion because interaction with the basic nitrogen as shown by the arrow implies formation of a seven-membered ring. A similar case has been discussed by Ullyot and Kerwin (34). The lack of activity of (XXXIII) is more difficult to explain on the basis of the concepts exposed thus far and other factors such as limitations imposed by molecular size as suggested by Ullyot and Kerwin (34), in connection with certain benzhydryl derivatives may be operating in this case. Other factors such as restricted rotation of bulky substituents on the EI-ion are very likely to affect



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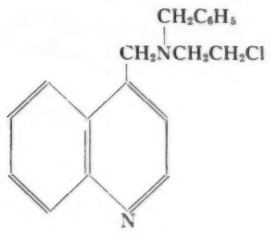

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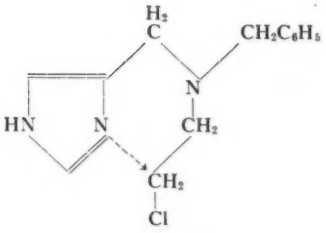

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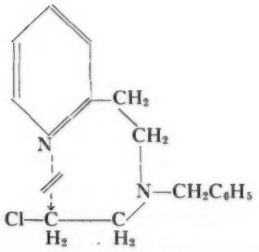
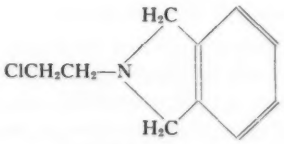


(XXXV)

adrenergic blocking ability but it would be premature at this time to dwell on such steric effects. Other N-benzyl-N-(β-haloalkylamines) carrying more exotic heterocyclic nuclei may best be treated as "Dibenamine" congeners and so do not require further attention.

It has been noted before that compounds in which the nitrogen bearing the β-haloalkyl group is part of a ring system exhibit no significant activity (34). Thus, 2-(β-chloroethyl)-isoindoline (XXXVI) and N-(β-chloroethyl)-2,3-dihydro-2,1-peribenzisoquinoline (XXXVII) are devoid of activity whereas N-(β-chloroethyl)-tetrahydroisoquinoline (XXXVIII) possesses very low



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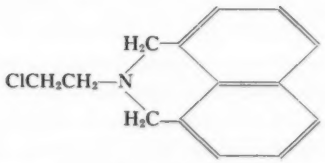
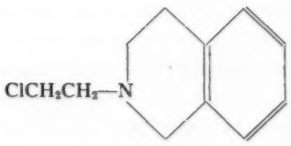
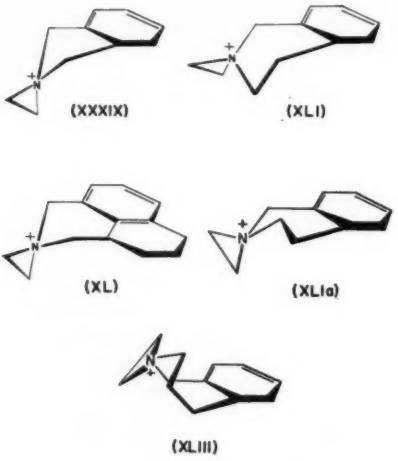


(XXXVII)

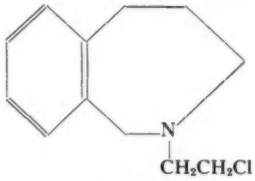


(XXXVIII)



activity in spite of the adherence of the corresponding EI-ions to the phenethylamine pattern with respect to the receptor. However, one fact is striking if the geometry of the corresponding EI-ions (XXXIX), (XL), (XLI), and (XLI*a*) is examined: the conformation of (XXXIX) and (XL) is that of *a rigid spirostructure in which the plane of the EI-ion intersects perpendicularly the center of the plane of the aromatic moiety, except for* (*XLI*), which allows some degree of movement to the planes with respect to each other because of the two possible conformations (boat and half-chair) of the tetrahydropyridine ring (XLI) and (XLI*a*). *Now, because of these rigid geometrical properties it is not possible to fit any of these three EI-ions on the receptor in a manner recalling the type of fitness ascribed to Dibenamine-EI-ion* (Fig. 2) in which the two planes can adopt any conformation with respect to each other. It is tempting therefore to ascribe biological inactivity of these cyclic compounds to the unfavorable geometry of their EI-ions, especially in view of the possibility for all other active series of β-haloalkylamines to avoid this type of rigid geometry. Concrete evidence for this interpretation was obtained[4] and can be briefly summarized as follows: the fact that in compound (XLI) the respective planes of the EI-ion and the aromatic ring need not intersect symmetrically as in the case of (XXXIX) and (XL) and that weak adrenergic blocking activity is obtained with this substance suggested that making the nitrogen part of a higher membered ring would allow still greater conformational freedom to the respective planes concerned. By doing so, it should become possible for a larger ring compound to adopt such a conformation as to lead to the proper "Dibenamine" type of fit on the receptor. The benzazepine derivative (XLII) was therefore synthesized[4] and, as can be seen in (XLIII), which represents but one of the possible conformations, the planes of the corresponding EI-ion and the aromatic ring have more conformational freedom with respect to one

another and make possible a proper fitness on the receptor. It is gratifying indeed that (XLII) was found to be a potent adrenergic blocking agent.[4]

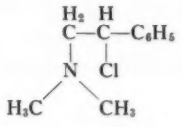


(XLII)

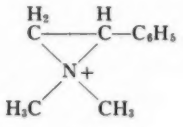
Similar results were obtained with 1,4-benzoxazepine and 2-benzazocine derivatives.[4] It seems clear therefore that conformational relationships between the planes of the EI-ions and the aromatic moieties of adrenergic blocking β-haloalkylamines must play a fundamental role in activity requirements. The mere fact of making the nitrogen of β-haloalkylamines part of a ring does not exclude blocking activity provided the selected ring has enough flexibility such as in reduced medium size rings.[4] A detailed study of such geometrical factors is under way and may lead to a better understanding of the geometrical requirements of the adrenotropic receptors.

N,N-dialkyl-l-chloro-2-phenethylamine

A remarkable class of blocking agents somewhat departing from the requisite phenethylamine pattern for high activity is exemplified by the prototype N,N-dimethyl-β-phenyl-β-chloroethylamine (XLIV) (27, 34). In the form of its



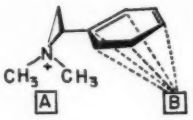
(XLIV)



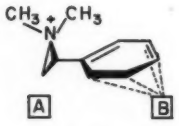
(XLV)

corresponding active EI-ion (34) (XLV), a favorable relationship of a cationic carbon with respect to the benzene ring is obtained only with regard to the first phase (see above, Fig. 2) in the establishment of blockade. As shown in (XLVI) and (XLVII), no better fit is obtained upon rearrangement to (XLVII), but assuming alkylation of (A) to take place in (XLVII) the formation of an ester linkage (XLVIII) makes possible ideal interaction of the benzene ring with center B, a fact which ought to favor equilibrium in the direction of blockade instead of desorption of the drug. It is of interest to note that the vicinal dimethylamino group in (XLVIII) should markedly influence the stability of the ester linkage at A because of its much smaller molecular size as compared to a dibenzylamino group. One might expect therefore that

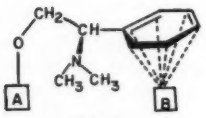
[4]Forthcoming publication as Part II of this series.



(XLVI)



(XLVII)



(XLVIII)

efficacious neighboring group participation in the hydrolysis of the ester linkage of (XLVIII) (see above in connection with ref. 5) should occur and lead to faster regeneration of the receptor surface with (XLV) than with "Dibenamine". Indeed this is found to be the case (24) and would tend to support the present interpretation of the activity of (XLV). On the basis of these arguments, the conclusion can be drawn that, for blocking activity to be observed, adherence to the phenethylamine pattern rule at the stage of the first phase (as in Fig. 2) may be sufficient provided a second phase of non-equilibrium blockade satisfying the steric requirements of the receptors is possible. Clearly, all other β-haloalkylamines of that series which carry a benzyl group on the nitrogen can be looked upon as "Dibenamine" congeners and no further comment is necessary.

Conclusion

The fact that the adrenergic blocking activity of most β-haloalkylamines can be interpreted on the basis of the above rationalizations may conceal some truth regarding the hypothetical properties on the epinephrine receptors. It is conceivable that through more extended but similar applications of chemical, isosteric, and conformational principles to a still greater number of β-haloalkylamines, much more fundamental knowledge of the biochemical nature and properties of the poorly defined receptors is to be gained in the future. On the basis of the evidence presented above, it has become possible for the first time to define with a reasonable degree of probability the nature of one of the active centers of the excitatory receptors. Of more immediate consequence is the presumption that the above interpretation can perhaps serve as an attractive working hypothesis for the elaboration of new and better therapeutic agents.

Acknowledgments

I am greatly indebted to the Canadian Life Insurance Officers Association for their generous support in the form of a fellowship. Appreciation is expressed to Professor Alfred Burger and Dr. Glenn E. Ullyot, who kindly read this manuscript and offered their valuable comments; a constructive criticism by the referee is also gratefully acknowledged. Thanks are also due to the National Research Council of Canada for the financial support of some phases of this work.

References

1. Axelrod, J., Aronow, L., and Brodie, B. B. J. Pharmacol. Exptl. Therap. **106**, 166 (1952).
2. Blackburn, S., Carter, E. G. H., and Phillips, H. Biochem. J. **35**, 627 (1941).
3. Chapman, N. B. and James, J. W. J. Chem. Soc. 2103 (1954).
4. Cohen, B., Artsdalen, E. R. V., and Harris, J. J. Am. Chem. Soc. **70**, 281 (1948); **74**, 1875 (1952).
5. Davis, W. and Ross, W. C. J. J. Chem. Soc. 3056 (1950).
6. Fruton, J. S., Stein, W. H., and Bergmann, M. J. Org. Chem. **11**, 559 (1946).
7. Fruton, J. S., Stein, W. H., Stahmann, M. A., and Golumbic, C. J. Org. Chem. **11**, 571 (1946).
8*a*. Golumbic, C., Fruton, S. C., and Bergmann, M. J. Org. Chem. **11**, 581 (1946).
b. Bartlett, P. D. *et al.* J. Am. Chem. Soc. **71**, 1415 (1949).
9. Goodlad, G. A. J. Biochim. et Biophys. Acta, **24**, 645 (1957).
10*a*. Graham, B. E. and Cartland, G. F. J. Pharmacol. Exptl. Therap. **81**, 360 (1944).
b. Mercier, F. and Mercier, J. Compt. rend. soc. biol. **138**, 658 (1944).
11. Graham, B. E. and Kuizenga, M. V. J. Pharmacol. Exptl. Therap. **94**, 150 (1948).
12. Gunn, J. A., Gurd, M. R., and Sacks, I. J. Physiol. **95**, 485 (1939).
13. Hambourger, W. E. and Jamieson, R. B. J. Pharmacol. Exptl. Therap. **58**, 53 (1936).
14. Hanby, W. E., Hartley, G. S., Powell, E. O., and Rydon, H. N. J. Chem. Soc. 519 (1947).
15. Harvey, S. C. and Nickerson, M. J. Pharmacol. Exptl. Therap. **109**, 328 (1953).
16. Heine, H. W., Miller, A. D., Barton, W. H., and Greiner, R. W. J. Am. Chem. Soc. **75**, 4778 (1953).
17*a*. Herriott, R. M., Anson, M. L., and Northrop, J. H. J. Gen. Physiol. **30**, 185 (1946);
b. Banks, T. E. *et al.* Biochem. J. **40**, 745 (1946).
c. Carpenter, F. H. *et al.* J. Am. Chem. Soc. **70**, 2551 (1948).
d. Ormsbee, R. A., Henriques, F. C., and Ball, E. G. Arch. Biochem. **21**, 301 (1949).
18. Hunter, W. T. *et al.* J. Org. Chem. **21**, 1512 (1956).
19. Kerwin, J. F., Ullyot, G. E., Fuson, R. C., and Zirkle, C. L. J. Am. Chem. Soc. **69**, 2961 (1947).
20. Kortüm, G. and Vogel, W. M. Z. Elektrochem. **59**, 16 (1955).
21. Lands, A. M. Am. J. Physiol. **169**, 11 (1952).
22*a*. Marsh, D. F. and Herring, D. A. J. Pharmacol. Exptl. Therap. **100**, 298 (1950);
b. Fellows, E. J., Macko, E., and Fendrich, A. J. J. Pharmacol. Exptl. Therap. **100**, 72 (1950).
23. Nickerson, M. J. Pharmacol. Exptl. Therap. **95**, 27 (1949).
24. Nickerson, M. Pharmacol. Revs. **9**, 246 (1957); Nature, **178**, 697 (1956).
25. Nickerson, M. and Goodman, L. S. Proc. Am. Federation Clin. Research, **2**, 109 (1945); Federation Proc. **5**, 194 (1946).
26. Nickerson, M. and Goodman, L. S. J. Pharmacol. Exptl. Therap. **93**, 40 (1948).
27. Nickerson, M. and Gump, W. S. J. Pharmacol. Exptl. Therap. **97**, 25 (1949).
28. Nickerson, M. and Nomaguchi, G. M. J. Pharmacol. Exptl. Therap. **107**, 284 (1953).
29. Oae, S. J. Am. Chem. Soc. **78**, 4030 (1956).
30*a*. Peters, R. A. Nature, **159**, 149 (1947).
b. Boursnell, J. C. *et al.* Biochem. J. **40**, 756 (1946).
31. Ross, W. C. J. J. Chem. Soc. 815 (1950).
32. Simonetta, M. and Winstein, S. J. Am. Chem. Soc. **76**, 18 (1954).
33. Streitwieser, A. Chem. Revs. **56**, 677 (1956).
34. Ullyot, G. E. and Kerwin, J. F. Medicinal chemistry. Vol. II. John Wiley & Sons, Inc., New York. 1956. p. 234.

PURINE SYNTHESIS IN SUSPENSIONS OF MUCOSA FROM THE SMALL INTESTINE OF THE RAT[1]

A. R. P. Paterson and S. H. Zbarsky

Abstract

Suspensions of the epithelial lining of the small intestine of the rat have been shown to be suitable for in vitro studies of the *de novo* pathway of purine biosynthesis. The mucosal suspensions incorporate radioactive precursors such as formate and glycine into the purines of the soluble nucleotides and the nucleic acids. The relative renewal rates of purines from glycine and formate indicate that the latter is incorporated principally by synthesis as opposed to exchange reactions. The acid-soluble adenine compounds showed the highest renewal rates in this system.

Introduction

In the development of biochemical knowledge concerning the pathways of purine nucleotide biosynthesis most of the experimental work has been done with pigeon liver enzymes and a lesser part with microbiological systems. The pigeon liver enzyme preparations have been mainly those concerned with the synthesis of inosinic acid and undoubtedly represent part of the enzyme system by which uric acid is synthesized. Inosinic acid (IMP) has been considered an obligatory intermediate in several concepts of purine nucleotide biosynthesis, adenine and guanine nucleotides being formed from IMP by amination and oxidation processes. It has been assumed that information derived from bacterial and avian studies is applicable to mammalian systems, but in mammals where other mechanisms for the excretion of nitrogen prevail, IMP formation is not such a prominent reaction and may not be an obligatory process in purine nucleotide biosynthesis. The participation of N-succinylo derivatives in the synthesis of adenylic acid (AMP) has been discussed recently by Carter (1), who suggested that the formylation of an intermediate such as amino-(N-succinylo-)carboxamidino-imidazole ribotide would lead to AMP by a route not involving IMP.

The present work is concerned with the selection of a *mammalian* tissue suitable for in vitro studies of purine biosynthesis. The epithelium of the small intestine of the rat was chosen for this work because of its high capacity for *de novo* purine synthesis in the intact animal (2). In the intestine formate and glycine are used more readily than adenine in the renewal of nucleic acid purines. In liver, by contrast, the utilization of adenine in the renewal of nucleic acid purines is favored over that of formate and glycine. Metabolically, intestinal mucosa is a very active tissue, having a high respiratory rate and being particularly rich in certain enzymes involved in metabolism of nucleic acid components (3,4). The very high rate of cell renewal in this tissue makes it particularly valuable in studies which attempt to relate nucleic acid metabolism to growth processes (5).

[1]Manuscript received March 6, 1958.
Contribution from the Department of Biochemistry, University of British Columbia, Vancouver 8, B.C.

In the present study a method was developed for preparing the mucosa of the small intestine of the rat as a suspension in Krebs–Ringer buffer solutions. In this form the tissue survives for several hours of incubation, respiring actively. Purine synthesis by the *de novo* route was shown to occur in such suspensions by measuring the incorporation of isotopic formate, glycine, and carbon dioxide into the acid-soluble (AS) and nucleic acid (NA) purine fractions. The renewal of the soluble adenine nucleotides was a prominent reaction being approximately six times greater than that of the guanine nucleotides. Specific activity relationships between the isotopic precursors and the isolated purines suggested that the acid-soluble nucleotides were intermediates in nucleic acid synthesis. The demonstration that rat intestinal mucosa is capable of a rapid synthesis of adenine and guanine in vitro suggests that this system may be profitably applied to a study of the intermediates involved in purine biosynthesis in mammalian tissue.

Experimental

A. General Procedure

The Preparation of Suspensions of Rat Intestinal Mucosa

Male rats[2] of the Wistar strain, weighing between 150 and 200 g., were used throughout this work. The animals were killed by a blow on the head and the small intestine was removed and immediately placed in cold, buffered saline (Krebs–Ringer phosphate-, or bicarbonate-buffered medium (6)). The organ was cut into 10 cm. segments which were flushed free of contents with the cold saline. The segments were split open, applied to a chilled glass plate, and blotted briefly with filter paper. The device illustrated in Fig. 1 greatly facilitated this step, splitting the segment and applying it to the plate in one operation. The mucosal epithelium was then scraped from the muscularis with the edge of a microscope slide (7) and placed in chilled, buffered saline. The tissue was suspended in the medium by gentle stirring and then recovered by centrifuging. After this washing had been repeated three or four times the mucosal suspension was fragmented by gently and repeatedly drawing the suspension into and expelling it from a 10-ml. syringe. The homogeneity of the suspension was improved by filtering it through one or two layers of gauze. All manipulations of the tissue were carried out as rapidly as possible, and solutions and containers were chilled by packing in cracked ice. The tissue suspension was diluted to such a volume that 3.0

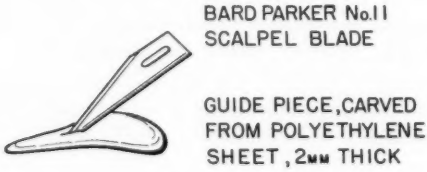


FIG. 1.

[2]Rats were obtained from the colony at the University of British Columbia.

ml., the amount used per Warburg vessel, contained approximately 30 mg. dry weight of tissue. Mucosal suspensions were incubated with various substrates for 3 hours (unless otherwise noted) at 37° C. in the Warburg respirometer. Respiration was followed when conditions permitted. After incubation, the tissue was recovered from the flask contents by centrifuging and the purines were recovered from the medium and tissue separately by the perchloric acid method of LePage (8).

Medium Fraction

The medium was made 0.2 *M* in perchloric acid (PCA) and the precipitated protein was spun down and discarded. The supernatant solution was then heated at 100° C. for 1 hour to hydrolyze any purine nucleotide compounds. Adenine and guanine were recovered from the hydrolyzate on small columns of Dowex-50 resin (8) and, after elution from the resin with 6 *N* HCl, were separated and purified by paper chromatography.

Uric acid was isolated by precipitation as the silver salt from the initial washings of the Dowex-50 columns. Solutions of uric acid were made alkaline with an excess of ammonium hydroxide and treated with an excess of magnesia mixture (9). After the mixture had been chilled in the refrigerator for several hours, the precipitate was filtered off and discarded. The filtrate was treated with an excess of ammoniacal silver nitrate solution (10), kept overnight in the refrigerator, and the silver purine precipitate which formed was recovered by centrifuging and washed with 0.5 *N* ammonium hydroxide. The precipitate was decomposed by heating with 3 *N* HCl and the precipitate of silver chloride so formed was discarded. The ultraviolet absorption spectra of such solutions of uric acid agreed very closely with the spectrum of pure uric acid. Aliquots of the uric acid solution were evaporated to dryness *in vacuo* and the residues suspended in water and mounted on aluminum planchets for C^{14} assay. Paper chromatography with a butanol – acetic acid solvent (11) did not alter the specific activity of uric acid.

Tissue Fraction

(a) Acid-soluble purines.—The tissue portions of the flask contents were each suspended in 3.0 ml. of cold 0.2 *M* PCA and allowed to stand for 15 minutes in the cold. This mixture was centrifuged to obtain the AS fraction, to which was added only the first of three successive washings of the tissue residue, made with 2.0 ml. portions of cold 0.2 *M* PCA. The AS fraction was hydrolyzed by heating in a boiling water bath for 1 hour and the purines were recovered from the hydrolyzates on Dowex-50 resin columns (8). After elution from the resin, adenine and guanine were separated and purified by paper chromatography.

(b) Nucleic acid purines.—The tissue residue from the preceding step was suspended in 3.0 ml. portions of 0.4 *M* PCA and heated in a gently boiling water bath for 1 hour to liberate the polynucleotide purines. The purines were recovered on Dowex-50 resin, then separated and purified by paper chromatography (8).

Center-Well Contents

Oxidation of the isotopic substrates was measured when phosphate buffer was used as the suspending medium by the determination of carbonate-C^{14} trapped in alkali in the center wells of the Warburg flasks. The alkali from the center wells was made up to a definite volume and suitable portions were removed for radiocarbon assay. "Carrier" carbonate was added to each sample before precipitation as barium carbonate in the C^{14} assay.

B. Methods

1. Chromatography and Spectrophotometry

Adenine and guanine were isolated from the hydrolyzed AS and NA extracts on Dowex-50 resin as mentioned above and after recovery from the resin were purified by paper chromatography. Chromatography in Wyatt's isopropanol–HCl–H_2O system (12) was adequate for the purification of NA purines since rechromatography in this and in other solvents did not change their specific activities. The AS purines, after an initial separation on chromatograms run in Wyatt's solvent, were rechromatographed in *n*-butanol–ethanol–water (11) to achieve radiochemical purity. The butanol solvent was found to leave, near the origin, ninhydrin positive material which accompanied acid-soluble adenine and guanine in the isopropanol solvent. In separating the purines from the glycine-C^{14} substrate, the butanol solvent has some advantage in the handling of guanine over the isoamyl alcohol – sodium phosphate system of Carter which has also been used for this purpose (13).

The purines were located by examining the chromatograms by ultraviolet light, the source of which was a germicidal lamp placed below a Corning 9863 glass filter mounted in a desk top. Outlining of the spots was greatly facilitated by using the filter as a surface against which to draw. This arrangement permitted the chromatograms to be scanned by transmitted light. Contact prints of purine chromatograms made with unfiltered light from the germicidal lamp were prepared routinely for record purposes and to aid in cutting out the purine areas for radiocarbon assay and spectrophotometry. For the detection of ultraviolet-absorbing areas the photographic print was much more sensitive than the visual examination. Uric acid spots were only faintly recorded with this photographic technique and, consequently, were located by chromatographing samples of authentic uric acid in guide lanes on the same paper. The guide lanes were cut off and the uric acid was located by spraying with ammoniacal silver nitrate reagent (11).

Disks were punched from purine-containing areas of the chromatograms and their purine contents were estimated by spectrophotometry after the measurement of radioactivity. The paper disks were cut into several pieces and extracted overnight at room temperature with 4.0 ml. of 0.1 *M* HCl. Before being read in the spectrophotometer, the extracts were shaken for at least 0.5 hour in a mechanical shaker. For estimation of the purine content of the extract the difference was measured between the optical densities at certain wavelengths (12) which are indicated in Table I, and by applying the appropriate molar extinction coefficient, the purine content of the extract was

TABLE I

ULTRAVIOLET ABSORPTION DATA USED IN THE DETERMINATION OF PURINES

Purine	λ_M	λ_R	Δ
Adenine	262.5 mμ	290 mμ	1.25×10^4
Guanine	248 mμ	300 mμ	1.02×10^4
Uric acid	284 mμ	310 mμ	1.13×10^4

NOTE: λ_M is the wavelength of maximum optical density.
λ_R is the reference wavelength, arbitrarily chosen at a point where the extinction is low, yet definite.
Δ is the difference between the molar extinction coefficients measured at λ_M and λ_R. These values were determined by the authors on carefully purified samples of the purines dissolved in 0.1 *N* HCl.

calculated. Purine determinations were made against blanks which consisted of extracts of disks cut from blank lanes of the same chromatogram. When this method of analysis was applied to filter paper disks containing 2–5 μg. of purine, replicate analyses varied by approximately 5% from their mean in the case of adenine and approximately 10% with guanine. With larger amounts of purines the variation in replicate analyses was smaller.

2. Radioactivity Measurements

Radioactivity was measured in windowless gas flow counters[3] connected to commercial scaling units. Two types of sample mountings were employed in the C^{14} assays; (*a*) samples contained in filter paper disks (26 mm. in diameter and usually cut from purine-containing areas on chromatograms) and, (*b*) "infinitely thick" samples of $BaCO_3$ (all carbonates were assayed in this form). In the work described below no comparisons were made between samples counted by different methods. In the particular type of counter employed it was noted that "infinitely thin" radioactive samples mounted on aluminum planchets or barium carbonate samples mounted in brass dishes (14) exhibited a declining counting rate with respect to time. In other words, replicate counts did not agree, successive counts becoming smaller. This was attributed to the build-up of a surface charge on the sample (15). With barium carbonate samples, this effect was abolished by insuring that the sample was thoroughly grounded through a conductive mounting. This was accomplished by impregnating the filter paper disk which supported the barium carbonate sample in the brass counting dishes with colloidal graphite[4] and by spreading out the carbonate sample until contact was made with the brass dish. If these precautions were observed replicate counts agreed within the expected limits of error. Filter paper samples did not display this variation in observed counting rates and excellent proportionality was obtained between C^{14}-activity and the amount of purine in the sample (up to 0.5 μM.). Formate-C^{14} was assayed in the form of filter paper samples to permit comparison with the purines, which were routinely counted in the same manner. To prevent a possible loss of activity through volatility caused by acid vapors

[3]Counter Model CFIS-2, purchased from Atomic Energy of Canada Limited.
[4]Filter paper disks were dipped two or three times in a 1:25 dilution of "Aquadag" (Acheson Colloids Company, Port Huron, Michigan, U.S.A.).

in the laboratory air, sodium formate-C^{14} samples were assayed on paper disks punched from filter paper that had been previously sprayed with 0.5 *M* K_2HPO_4 (16).

Estimate of errors.—The statistical errors inherent in the counting procedure were estimated as the percentage errors of the net activity and unless otherwise noted are less than 5%.

3. Substrates and Labelled Compounds

Sodium formate-C^{14}.—Radioactive sodium formate obtained from Atomic Energy of Canada Ltd. was shown by paper chromatography (16) and radioautography to have no detectable amounts of radioactive impurities. The specific activity of formate was 1.59×10^6 c.p.m. per micromole and the material was used routinely in 2.25 μM. portions per Warburg flask unless otherwise noted. To permit comparison with the radioactivity of carbon dioxide derived from the biological oxidation of formate-C^{14} the specific activity of the stock formate-C^{14} was determined after oxidation to carbon dioxide by the mercuric sulphate method (17). The specific activity measured on "infinitely thick" samples was found to be 0.32×10^6 c.p.m. per micromole.

Glycine-1-C^{14}.—Carboxyl-labelled glycine was prepared by the reaction of isotopic cyanide with N-chlormethylphthalimide (18). The isotopic cyanide for this reaction was synthesized from barium carbonate-C^{14} by the method of McCarter (19) as modified by Jeanes (20). Paper chromatograms of the labelled glycine produced in this way showed only one ninhydrin-positive spot which coincided with the location of radioactivity as determined by radioautography. Samples mounted on filter paper indicated a specific activity of 2.64×10^5 c.p.m. per micromole.

Sodium bicarbonate-C^{14}.—Sodium bicarbonate-C^{14} was prepared by trapping C^{14}-carbon dioxide generated from barium carbonate-C^{14} in the stoichiometric amount of sodium hydroxide. Bicarbonate, having a specific activity of 1.12×10^6 c.p.m. per micromole was prepared in this manner.

Glycinamide hydrochloride.—The ammonolysis of glycine ethyl ester hydrochloride was used to prepare glycinamide hydrochloride which was purified by recrystallization from a methanol–ether solution (21). On paper chromatograms the product showed a single yellow spot with ninhydrin reagent.

4-Amino-5-imidazolecarboxamidine dihydrochloride.—This compound was synthesized by the method of Shaw (22) and was crystallized twice from methanol–ether solution.

Results

Characteristics of the Intestinal Mucosa Suspension

The mucosal suspensions consisted of small fragments of tissue ranging in size from clumps of a few cells to sheets of cells approximately 5 mm.^{2} in area. These suspensions respired actively for 3–4 hours at gradually declining rates. Q_{O_2} values, which averaged approximately 7 (values of 11 and 12 were also recorded), were comparable with the range of values Dickens and Weil-Malherbe (7) reported for mucosa from various regions of the small intestine.

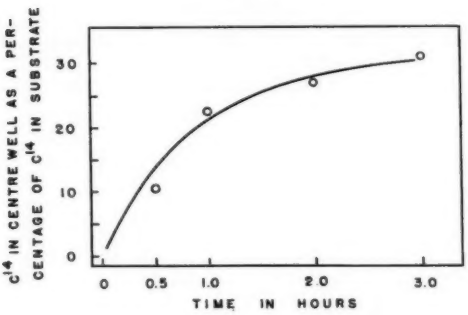


FIG. 2.

The addition of aureomycin to the medium (10 p.p.m.) had no measurable effect on oxygen uptake in these preparations.

Bacterial contamination was small in these preparations as bacterial counts did not exceed 2×10^6 bacteria per milliliter in the washed preparation and in most cases were considerably lower. Aureomycin (10 p.p.m.) had the effect of lowering the bacterial counts and was used routinely in the medium as a safeguard against bacterial growth during incubation. Bacterial contamination was considered to exert no significant influence on the purine metabolism of the preparations since homogenates of mucosa were uniformly inactive with respect to the uptake of formate into the purines. The observation that oxygen consumption decreased with time also supports this conclusion. Analysis of the contents of the center wells of the respirometer flasks showed that the mucosal suspension oxidized formate readily. In Fig. 2 the radioactivity recovered as carbonate from the center wells is expressed as a percentage of the total radioactivity originally present and is plotted against time of incubation. Glycine-1-C^{14} was oxidized to only a very slight extent (approximately 0.2%) under these conditions.

Purine Synthesis in Mucosal Suspensions

The purines of the preparations were found to be radioactive after incubation with formate-C^{14}, but before significance could be attached to this observation it was essential to establish the radiochemical purity of these compounds. This was done by comparing the specific activities of the isolated purines before and after rechromatography in different solvents. The specific activities of the NA purines were unaltered by rechromatography. The acid-soluble purines, initially separated in Wyatt's solvent (12), were rechromatographed in butanol–ethanol–water (11). This procedure generally increased specific activities, resulted in more consistent data, and caused duplicate determinations to agree more closely.

From these findings it was concluded that the C^{14}-content of the isolated purines was real and not due to accompanying radioactive contaminants.

TABLE II

A COMPARISON OF FORMATE-C^{14} AND GLYCINE-1-C^{14} INCORPORATION BY THE ACID-SOLUBLE PURINES

Flask	Substrates	Specific activity (c.p.m. per μM.)$\times 10^{-3}$	
		AS adenine	AS guanine
1	C^{14}-Formate	28.1	6.13
2	C^{14}-Formate + glycine	34.5	5.92
3	C^{14}-Glycine	1.01	0.27
4	C^{14}-Glycine + formate	1.14	0.27

NOTE: Medium: Krebs–Ringer carbonate.
Substrates, amount per flask: glycine-1-C^{14}, 8.5 μM. (2.5×10^6 c.p.m.); sodium formate-C^{14}, 9.5 μM. (19.7×10^6 c.p.m.); glycine, 10 μM.; sodium formate, 10 μM.

This indicated that *de novo* purine synthesis was occurring, but did not exclude the possibility that formate-C^{14} was being incorporated by exchange reactions.

To test this possibility, the experiment outlined in Table II was carried out. In this experiment, the incorporation of formate-C^{14} into the acid-soluble purines of a mucosal suspension was compared with that of glycine-C^{14}, a purine precursor which is not known to undergo exchange reactions. The C^{14}-formate used as substrate had a specific activity of approximately 7 times that of the C^{14}-glycine. The results, shown in Table II, suggest that *de novo* synthesis had occurred. Assuming no dilution from endogenous sources, the formate should give rise to purines approximately 14 times as active as those derived from glycine, since two molecules of formate are incorporated in the purine ring (positions 2 and 8) for every molecule of glycine (positions 4, 5, and 7). From a comparison of the specific activities of the adenine derived from these substrates it appeared that glycine was used in approximately one half of the amount expected from the observed incorporation of formate. The results with guanine were similar, and indicated that approximately three molecules of formate were used for each glycine molecule instead of the theoretical 2:1 ratio. In a similar experiment, two molecules of formate were apparently used for each molecule of glycine in the formation of AS adenine and in this experiment it was shown also that significant amounts of bicarbonate-C^{14} were incorporated into the AS purines.

The simultaneous incorporation of three purine precursors—glycine, formate, and bicarbonate—strongly suggests that *de novo* synthesis was occurring in the soluble purine nucleotide fraction. The departure from the theoretical 2:1 ratio for the incorporation of formate and glycine might be attributed to the dilution of radioactivity of the substrates by endogenous pools of these metabolites or possibly to some inosinic acid transformylase activity (23). This enzyme system from pigeon liver has been shown to catalyze an exchange reaction between formate and the 2-position of inosinic acid. Such a reaction could place more isotope from formate in position 2 of the purine ring in adenosine and guanosine phosphates than would be derived by synthesis alone.

TABLE III

A COMPARISON OF REPLICATE SAMPLES

Warburg vessel	Specific activity (c.p.m. per μM.)$\times 10^{-2}$				% of total formate-C^{14} in center well
	Acid-soluble fraction		Nucleic acid fraction		
	Adenine	Guanine	Adenine	Guanine*	
1	181	17.2	1.83	0.78	18.6
2	192	15.1	1.83	0.68	18.3
3	181	16.1	1.62	0.64	18.7
4	151	14.2	1.51	0.59	18.7
5	170	12.8	1.81	0.68	19.0
Average	175 $\pm$13.8	15.1 $\pm$1.26	1.72 $\pm$0.13	0.67 $\pm$0.061	18.7

NOTE: Medium: Krebs–Ringer phosphate.
Subtrates: sodium formate-C^{14}, 2.25 μM. (3.58×10^6 c.p.m.) per flask.
Solvent system for chromatography: isopropanol–HCl.
C^{14} assay: *error of net activity less than $\pm$ 9%.
Values for NA purines are averages of duplicate analyses.

Some Characteristics of Purine Synthesis in Suspensions of Intestinal Mucosa

The existence of differences in the metabolic activity of the mucosa from various regions of the small gut was noted by Dickens and Weil-Malherbe (7), who measured these differences in terms of Q_{O_2}. Therefore, it is apparent that the mucosal suspension must be homogeneous when a series of replicate samples are to be taken. The data of Table III illustrate the reproducibility of results obtained with different samples of the same mucosal suspension. The variations, although less than 15% of the mean, indicate that the homogeneity of the preparation could have been improved. Generally better agreement between duplicates was achieved in subsequent experiments by more effectively fragmenting and filtering the tissue suspensions. The use of a chelating agent (24) may facilitate fragmenting of the mucosa and thereby avoid the mechanical damage which would increase with additional manipulation during preparation.

The data of Table III demonstrate that renewal of the nucleic acid purines also occurred in these preparations. Typical values for the AS and NA purines are arranged for comparison in Fig. 3. Isotopic formate was used as a substrate in all cases and Q_{O_2} values were similar. The acid-soluble purines were highly labelled, the adenine and guanine of this fraction being approximately 40 and 14 times more active, respectively, than the corresponding nucleic acid purines. AS adenine, which consistently displayed the highest renewal rates, was six or more times as active as AS guanine. Nucleic acid adenine was usually two to three times as active as nucleic acid guanine.

The suspending medium from which the tissue was separated following incubation was analyzed separately for its purine content. Uric acid occurred in relatively large amounts (60–70 μg. per flask) in the medium, and adenine and guanine were also present in amounts 5–10 times larger than in the acid-soluble fraction. In Table IV the specific activities of the purines in the medium are

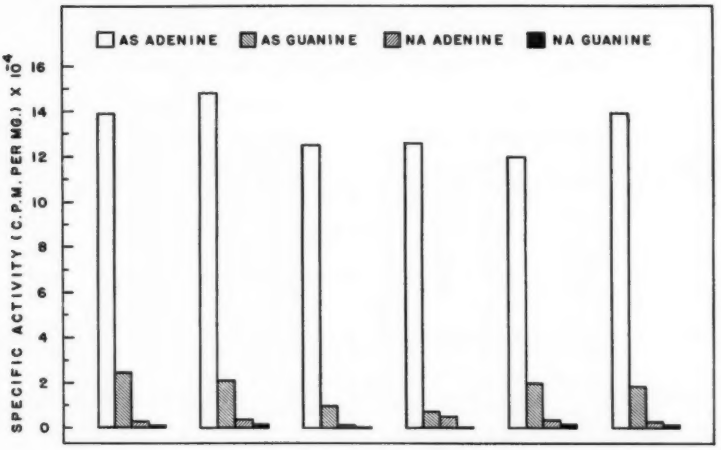


FIG. 3.

TABLE IV

A COMPARISON OF THE SPECIFIC ACTIVITIES OF ACID-SOLUBLE AND NUCLEIC ACID PURINES AND THE PURINES OF THE MEDIUM

Period of incubation, hours	Specific activity (c.p.m. per μM.)$\times 10^{-2}$							% of formate-C^{14} in center well
	AS adenine	AS guanine	NA† adenine	NA† guanine	Med.*** adenine	Med. guanine	Med.** uric	
0.5	75.2	11.2*	34.1	15.6	0.54	Inactive	—	10.2
1.0	83.0	12.4	47.0	16.8	0.67	Inactive	4.55	23.3
2.0	102.7	9.6	75.2	20.8	0.95	Inactive	4.38	27.0
3.0	140.7	13.3	76.5	19.1	1.22	Inactive	4.89	31.4

NOTE: Medium: Krebs–Ringer phosphate.
Substrate: sodium formate-C^{14}, 2.25 μM. (3.58×10^6 c.p.m.) per flask.
Solvent system for uric acid chromatography was *n*-butanol–ammonia (11).
C^{14} assay: *error of net activity less than $\pm 10\%$,
**error of net activity less than $\pm 15\%$,
***error of net activity less than $\pm 20\%$.
†The specific activities of the nucleic acid purines in this experiment are higher than in others. The values were not changed on rechromatography.

compared with those of the other fractions of the preparation. The adenine of the medium fraction was only slightly radioactive and the guanine was non-radioactive, suggesting that the purines in the medium arose from the breakdown of tissue during incubation. Cytosine (non-radioactive) was also found on chromatograms of the medium fraction. The specific activities of uric acid and adenine of this fraction were approximately 3% and 1%, respectively of the AS adenine. The origin of the C^{14}-uric acid in the medium is a matter of conjecture. It may have been derived from the purines of cells that succumbed during incubation since intestinal mucosa is rich in xanthine oxidase (4). The C^{14}-uric acid may have been produced exogenously from purine anabolites of formate which had "leaked out" of the mucosal cells or, possibly, was produced endogenously and then passed to the medium.

Formate Incorporation with Respect to Time

A typical plot of the renewal rates of the AS and NA purine fractions from formate is shown in Fig. 4. There was some variation in the shape of such time curves in four similar time studies but their averaged results indicated an approximately linear uptake of formate with respect to time. The data of Fig. 4 indicate a declining rate of incorporation of formate after 4 hours of incubation. The mucosal suspension was deteriorating at this time (falling pH and rate of respiration). In this particular experiment it was shown that aureomycin had no effect on purine renewals. All values shown are the averages of two incubations, one with aureomycin and one without. Since there were no significant differences in the specific activities of the purines of these pairs they were treated as duplicates and averaged. In subsequent experiments incubation periods were not extended beyond 3 hours since the preparations were deteriorating beyond that time and also because the possibility of bacterial growth increased with time. It will be noted in Fig. 4 that there is a time lag in the appearance of radioactivity in the nucleic acid purines. This was typical of the several rate experiments and may be another manifestation of the observation made with intact animals that nucleic acid precursors pass through the acid-soluble nucleotide pool before entering the nucleic acid fraction (2, 25).

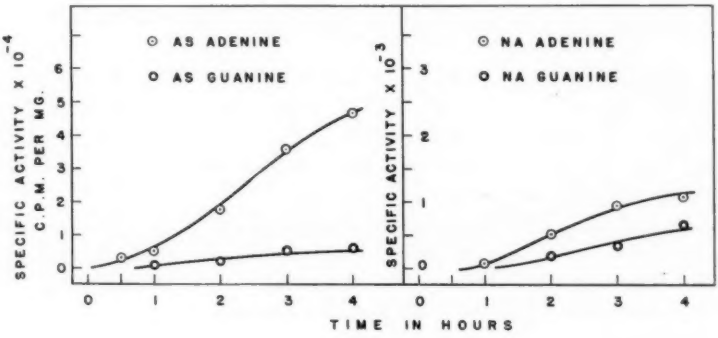


FIG. 4.

The Effect of Additives on Purine Synthesis

An attempt was made to influence the incorporation of radioactive formate into the purines of the mucosal suspension by adding to the incubation mixture certain compounds which were known to be involved in purine biosynthesis. Glycine and glutamine, known to contribute to the purine ring, were tested in this way, as were glycinamide and 4-amino-5-imidazolecarboxamidine. The last compound was considered likely to influence purine biosynthesis because of its structural resemblance to adenine. The ribotide of glycinamide and its formyl derivative have been implicated as intermediates in inosinic acid biosynthesis (26). If they were to have any effect, glycine, glutamine, and

TABLE V

THE EFFECT OF VARIOUS ADDED COMPOUNDS ON PURINE BIOSYNTHESIS FROM FORMATE-C^{14}

Compound added				Specific activity (c.p.m. per μM.)$\times 10^{-2}$			
				Acid-soluble fraction		Nucleic acid fraction	
Glycine	Glycinamide	Glutamine	4-Amino-5-imidazole carboxamidine	Adenine	Guanine	Adenine	Guanine
Expt. 1							
–	–	–	–	205	35.9	6.40	3.02
–	–	10 μM.	–	172	lost	3.36	2.13
10 μM.	–	–	–	168	39.2	3.75	2.13
10 μM.	–	10 μM.	–	144	27.0	2.96	1.83
–	–	10 μM.	–	184	38.7	5.11	1.67
–	10 μM.	10 μM.	–	241	44.8	4.45	2.13
Expt. 2							
–	–	–	–	228	31.6	6.60	3.34
–	–	10 μM.	–	250	30.7	5.40	3.64
Expt. 3							
–	–	–	–	9.3	1.51	2.70	3.19
–	–	–	2 μM.	13.5	3.17	2.70	1.51

NOTE: Medium: Expts. 1 and 2—Krebs–Ringer bicarbonate; Expt. 3—Krebs–Ringer phosphate. Substrate: Sodium formate-C^{14}, 2.25 μM. (3.58×10^6 c.p.m.) per flask.

glycinamide were expected to exert a stimulating influence on the uptake of formate-C^{14} by the purines. A lowering in formate-C^{14} uptake was expected with 4-amino-5-imidazolecarboxamidine if this compound was to be incorporated into the purines, since it would require the addition of only one formate molecule to complete the purine ring.

The data of Table V indicate that the addition of any of the above compounds to the medium did not significantly influence purine synthesis in the mucosal system. The variations observed in these data could be explained by the relative inhomogeneity of the suspensions of mucosa.

Purine Synthesis in Homogenates

The renewal of AS and NA purines from formate-C^{14} was also investigated in homogenates of mucosa. The homogenates were prepared in all-glass homogenizers of the Potter–Elvehjem type in a medium of the following composition: potassium chloride, 0.13 *M*; potassium phosphate buffer (pH 7.4), 0.03 *M*; magnesium chloride, 0.003 *M*; potassium bicarbonate, 0.04 *M*; glucose, 0.01 *M*. The homogenates respired at a very low rate and uniformly failed to incorporate formate into adenine and guanine of either the AS or NA fractions. Uric acid, also not radioactive, was found in large amounts (approximately 400 μg. per flask), a sixfold increase over the amount ordinarily found in the suspensions. Wenner *et al.* have noted that kidney, heart, and liver homogenates have an absolute requirement for diphosphopyridine nucleotide

(27). Following their suggestions, the mucosal homogenates were fortified with DPN, 0.0002 *M*; cytochrome *c*, 0.00004 *M*; ATP, 0.0002 *M*. In addition, the following compounds were included in the incubation mixture to ensure an adequate supply of components for *de novo* purine synthesis, ribose-5-phosphate, 0.005 *M*; glutamine, 0.01 *M*; glycine, 0.01 *M*. These additives had no effect on purine synthesis and caused only a slight increase in the oxygen consumption of the homogenates. The inactivity of the homogenates with respect to purine synthesis was probably related to the inability of the system to maintain respiration.

Incorporation of Formate-C^{14} into the Purines of Intestinal Mucosa in the Intact Rat

The distribution of formate-derived radioactivity in the AS and NA purines of intestinal mucosa from the intact rat is included for comparison with the results of the in vitro experiments. The intestinal tissue used in this experiment was obtained from male Wistar rats bearing the Novikoff hepatoma. These animals received a single dose of formate-C^{14} administered subcutaneously on the back and were killed 24 hours later. This experiment, which is summarized in Table VI, did not show the large differences between the specific activities of the AS and NA fraction that were observed in the in vitro experiments. Probably this is due to the 24-hour interval between the administration of the isotope and the death of the animal, during which time the specific activities of the acid-soluble purines had undoubtedly fallen below their maximum values and were approaching those of the nucleic acid purines. Time studies of purine renewals in intact animals, such as those of Bennett (25) and Hurlbert *et al.* (28) indicate this trend. The ratio of the specific activities of AS adenine to AS guanine was approximately 1.4, whereas, in the in vitro experiments this value was approximately 6. This discrepancy may indicate some impairment of guanine synthesis in the in vitro system. The ratios of the specific activities of NA adenine to NA guanine were similar to those reported by Drochmans *et al.* (29).

TABLE VI

THE INCORPORATION OF FORMATE-C^{14} BY THE PURINES OF INTESTINAL MUCOSA IN THE INTACT RAT

	Specific activities in c.p.m. per $\mu M. \times 10^{-2}$			
	Acid-soluble fraction		Nucleic acid fraction	
Rat	Adenine	Guanine	Adenine	Guanine
1 (tumor)	56.2	44.9	30.1	30.6
2 (control)	48.8	38.2	28.5	32.2
3 (tumor)	60.2	42.9	38.6	41.8
4 (control)	56.5	57.0	45.5	49.9

NOTE: Each horizontal row of values was obtained from one rat which received subcutaneously 225 $\mu M.$ sodium formate-C^{14} (3.58×10^6 c.p.m.) Animals were killed 24 hours later by bleeding under ether anaesthesia. Intestinal mucosa was placed in 2% perchloric acid and the purines prepared in the usual fashion.

Discussion

Earlier studies of the metabolism of nucleic acid purines in intestine have been confined to observations of the incorporation of isotope from labelled precursors in the intact animal. Of the viscera of the rat, the intestine displays the highest rate of synthesis of DNA and RNA purines from small molecule precursors such as formate, glycine, and ammonia (2). Relative to liver, intestine has a smaller ability to incorporate preformed purines into the nucleic acids (2, 29). In the intact rat and mouse the small intestine is perhaps the most active visceral tissue in effecting the conversion of radioactive adenine to acid-soluble purine nucleotides (2, 25, 30). The prominence of nucleic acid synthesis in this tissue is probably related to the very high mitotic activity.

The vigorous purine synthesis demonstrated in the intestine of the intact animal manifested itself in the mucosal suspension in vitro by the synthesis of both acid-soluble and nucleic acid purines. That the radioactivity associated with the purines of the preparation was truly incorporated in the purine molecule, and was not due to radioactive contaminants, was established by demonstrating the radiochemical purity of the isolated purines.

The incorporation of the early purine precursors, glycine, formate, and bicarbonate, into the purines of the mucosal suspensions was regarded as indicating that purine biosynthesis was occurring. The relative amounts of glycine and formate incorporated into the AS purines were sufficiently close to the theoretical proportions of 2:1 to exclude a major incorporation of formate by an exchange reaction. A comparison of the relative incorporation of labelled formate and glycine is complicated by the endogenous occurrence of these compounds (31, 32). The observation that the incorporation of glycine relative to that of formate was less than theoretical in the present experiments may be due to dilution of the labelled glycine by endogenous glycine.

The rapid renewal of the AS purines which occurs in vivo was apparent during the incubation of the mucosal suspensions. Acid-soluble adenine and guanine were found to be 43 and 14 times more active, respectively, than the corresponding nucleic acid purines. In the mucosal suspension adenine renewals from formate-C^{14} were more rapid than those of guanine. LePage found the reverse to be true in suspensions of mouse tumor and liver cells with glycine-2-C^{14} as the labelled substrate (8). Totter and Best (33) have investigated formate incorporation into the nucleic acids of rabbit bone marrow cells in vitro and report specific activities for RNA adenine 3–5 times greater than those of RNA guanine. The reason for the divergence between the rates of adenine and guanine synthesis in these reports is not apparent, but may be due in part to differences in the sizes of the pools of endogenous nucleotides.

Time studies such as that shown in Fig. 4 demonstrated that the AS purines are synthesized at an approximately linear rate and emphasize the difference between the rate of synthesis of adenine and guanine.

The possibility was investigated that the addition of certain compounds to the incubation mixture could influence formate incorporation in such a way

as to indicate an involvement of the additive in purine synthesis. However, none of the compounds added affected this process. It is now believed that free glycinamide is not involved in purine synthesis, present indications being that glycine, by interaction with 5-phosphoribosylamine, enters the biosynthetic pathway as glycinamide ribotide (34). The negative findings with glycinamide in the mucosal system are compatible with these concepts. Abrams has shown that 4-amino-5-imidazolecarboxamide has no effect on formate uptake by bone marrow slices (35). The amidine of this compound was tested in the mucosal suspension because of its structural similarity to possible adenine precursors and because of the indication that it may have a metabolic function in certain protozoa (36).

Suspensions of intestinal mucosa have not hitherto been utilized for in vitro studies of purine metabolism (37). The demonstration that both acid-soluble and polynucleotide purines are rapidly synthesized in the mucosal suspension suggests that this preparation may be of value in studies of purine metabolism and nucleotide biosynthesis. In this field comparisons between avian and mammalian systems are distinctly needed and intestinal mucosa suspensions have a useful function in this respect.

Acknowledgments

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References

1. Carter, C. E. Ann. Rev. Biochem. **25**, 123 (1956).
2. Brown, G. B. and Roll, P. M. *In* The nucleic acids. Vol. II. *Edited by* E. Chargaff and J. N. Davidson. Academic Press, Inc., New York. 1955. p. 341.
3. Allfrey, V. G., Mirsky, A. E., and Stern, H. Advances in Enzymol. **16**, 441 (1955).
4. Westerfeld, W. W. and Richert, D. A. Proc. Soc. Exptl. Biol. Med. **71**, 181 (1949).
5. Stevens, C. E., Daoust, R., and Leblond, C. P. Can. J. Med. Sci. **31**, 263 (1953).
6. Cohen, P. P. *In* Manometric techniques and tissue metabolism. *By* W. W. Umbreit, R. H. Burris, and J. F. Stauffer. Burgess Publishing Co., Minneapolis, Minn. 1949. p. 118.
7. Dickens, F. and Weil-Malherbe, H. Biochem. J. **35**, 7 (1941).
8. LePage, G. A. Cancer Research, **13**, 178 (1953).
9. Hawk, P. B., Oser, B. L., and Summerson, W. H. Practical physiological chemistry. 13th ed. The Blakiston Co., Inc., New York. 1954.
10. Graves, S. S. and Kober, P. A. J. Am. Chem. Soc. **37**, 2430 (1915).
11. Berry, H. K., Sutton, H. E., Cain, L., and Berry, J. S. Univ. Texas Publ. No. 5109, 23 (1951).
12. Wyatt, G. R. *In* The nucleic acids. Vol. I. *Edited by* E. Chargaff and J. N. Davidson. Academic Press, Inc., New York. 1955. p. 243.
13. Carter, C. E. J. Am. Chem. Soc. **72**, 1466 (1950).
14. Zbarsky, S. H. and Wright, W. D. Can. J. Med. Sci. **31**, 151 (1953).
15. Reid, A. F. and Robbins, M. C. Science, **116**, 148 (1952).
16. Kennedy, E. P. and Barker, H. A. Anal. Chem. **23**, 1033 (1951).
17. Weinhouse, S. and Friedmann, B. J. Biol. Chem. **197**, 733 (1952).
18. Sakami, W., Evans, W. E., and Gurin, S. J. Am. Chem. Soc. **69**, 1110 (1947).
19. McCarter, J. A. J. Am. Chem. Soc. **73**, 483 (1951).
20. Jeanes, J. K. Science, **118**, 719 (1953).
21. Yang, P. S. and Rising, M. J. Am. Chem. Soc. **53**, 3183 (1931).
22. Shaw, E. J. Biol. Chem. **185**, 439 (1950).

23. BUCHANAN, J. M., LEVENBERG, B., FLAKS, J. G., and GLADNER, J. A. *In* Symposium on amino acid metabolism. *Edited by* W. D. McElroy and H. B. Glass. The Johns Hopkins Press, Baltimore, Md. 1955. p. 743.
24. ANDERSON, N. G. Science, **117**, 627 (1953).
25. BENNETT, E. L. and KRUECKEL, B. J. Biochim. et Biophys. Acta, **17**, 503 (1955).
26. GOLDTHWAIT, D. A., PEABODY, R. A., and GREENBERG, G. R. J. Biol. Chem. **221**, 555 (1956).
27. WENNER, C. E., DUNN, D. F., and WEINHOUSE, S. J. Biol. Chem. **205**, 409 (1953).
28. HURLBERT, R. B. and POTTER, V. R. J. Biol. Chem. **209**, 1 (1954).
29. DROCHMANS, P., MARRIAN, D. H., and BROWN, G. B. Arch. Biochem. Biophys. **39**, 310 (1952).
30. MARRIAN, D. H. Biochim. et Biophys. Acta, **13**, 282 (1954).
31. WEINHOUSE, S. and FRIEDMANN, B. J. Biol. Chem. **210**, 423 (1954).
32. SOMMERVILLE, R. L. Master of Science thesis, Univ. of British Columbia. 1957.
33. TOTTER, J. R. and BEST, A. N. Arch. Biochem. Biophys. **54**, 318 (1955).
34. GOLDTHWAIT, D. A., GREENBERG, G. R., and PEABODY, R. A. Biochim. et Biophys. Acta, **18**, 148 (1955).
35. ABRAMS, R. and GOLDINGER, J. M. Arch. Biochem. Biophys. **35**, 243 (1952).
36. AARONSON, S. and NATHAN, H. A. Biochim. et Biophys. Acta, **15**, 306 (1954).
37. PATERSON, A. R. P. and ZBARSKY, S. H. Biochim. et Biophys. Acta, **18**, 441 (1955).

THE METABOLISM OF ANIMAL TISSUES CULTIVATED IN VITRO

III. AMINO ACID METABOLISM OF STRAIN L CELLS IN COMPLETELY SYNTHETIC MEDIA[1]

ARTHUR E. PASIEKA, HELEN J. MORTON, AND JOSEPH F. MORGAN

Abstract

Strain L cells, of mouse fibroblastic origin, have been cultivated in vitro in completely synthetic medium M 150 and in various modifications of this medium. The amino acid changes in the nutrient medium during cell cultivation have been studied by paper chromatography. A characteristic pattern of amino acid uptake and accumulation in the medium has been found. No change in the alanine concentration was observed but omission of alanine from the culture medium resulted in its accumulation in appreciable amounts. Omission of glutamic acid did not alter the pattern of amino acid changes by the cells. Omission of glutamine increased the uptake of amino acids and prevented amino acid accumulation. Omission of both glutamic acid and glutamine resulted in a virtual cessation of amino acid changes in the culture medium. Strain L cells decreased the adenine content of the medium and produced small amounts of hypoxanthine. These changes were not affected by alterations in the amino acid content of the medium. Omission of glutamic acid and glutamine from the culture medium did not cause an appreciable decrease in cell population or apparent degeneration of the cultures over a 30-day period.

Introduction

Previous studies from this Laboratory have shown that characteristic patterns of amino acid decrease and accumulation occur during the cultivation of chick embryonic heart (1) and other freshly explanted tissues (2) in completely synthetic medium M 150 (3, 4). These patterns of amino acid changes in the culture medium have been shown to be different with each type of tissue cultivated. Extension of these experiments to include cell strain cultures, as distinct from freshly explanted tissues, has now been carried out. The present communication reports the pattern of amino acid changes observed during cultivation of strain L cells in synthetic media and emphasizes the importance of glutamic acid and glutamine in regulating the amino acid metabolism of this cell strain.

Materials and Methods

Tissue Culture Procedures

All cultures employed in this study were prepared from strain L cells. This cell line was obtained from Dr. W. R. Earle, of the National Cancer Institute, Bethesda, in April, 1954, and for the past 3½ years has been maintained by continuous cultivation in this Laboratory. During this period, synthetic and semisynthetic media have been alternated as nutrients. The usual schedule has been to cultivate the cells for 6 weeks in synthetic medium M 150 (3, 4) or M 416 (5), followed by a 1- to 2-week cultivation period in which the synthetic medium was supplemented by the addition of 10% horse serum (Difco).

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Contribution from the Biochemical Research Laboratories, Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Ontario.

Use of this system of alternating nutrients made it possible to maintain the stock cultures in T-30 flasks (6) at a fairly constant rate of cell multiplication.

Routine subcultures were made at intervals of 10 to 14 days, depending on the density of the cell population. The stock cultures were examined microscopically and only cultures free from apparent degeneration were selected. The medium was withdrawn by capillary pipette, 5.0 ml. of medium M 416 added, and the cell layer washed by a gentle rotation of the flask. The washing medium was removed and 5.0 ml. of fresh M 416 added. The cell layer was then scraped gently from the surface of the flask with a capillary pipette, and the resulting cell suspension aspirated several times with a fine capillary pipette to break up cell clumps. A 2.0-ml. aliquot of this suspension was then used to seed a new T-30 flask. For experimental purposes, the cell suspension from several comparable stock cultures was pooled, mixed, and the desired number of new T-30 flasks seeded.

Groups of cultures were selected for experiments after cultivation in synthetic medium M 150 for approximately three weeks, with twice-weekly changes of medium. At this time, total protein per flask ranged between 8 and 12 mg., wet weight, as determined by a modification (7) of the Lowry protein method (8). At this stage of development, a monolayer of cells covered approximately three-fourths of the surface of the flasks. Heavy cell populations of this order were found necessary to bring about detectable changes in the nutrient media, but moderate variations in total cell number were not found to affect the final determinations. Suitable cultures selected for experiments then received the appropriate test medium for a period of 3 weeks (six renewals of fluid). During this time, the used media aspirated from the cultures before each renewal were pooled and used for chromatographic analyses. All cultures were examined microscopically before the fluid renewals. The majority of the experiments were discontinued after one test medium, but in certain instances cultures were treated with a series of experimental media, receiving each in turn for a period of 3 weeks.

The basic medium used on the experimental cultures (M 150) was modified by the omission of selected amino acids, as shown in Table I. All media were prepared at frequent intervals and sterilized by passage through UF fritted glass filters.

TABLE I

AMINO ACID CONTENT OF VARIOUS SYNTHETIC TISSUE CULTURE MEDIA EMPLOYED*

Medium No.	Amino acid content
M 150	Complete supplement of 21 amino acids, including glutamic acid and glutamine
M 746	Glutamic acid omitted
M 762	Glutamine omitted
M 856	Glutamic acid and glutamine omitted
M 894	Glutamic acid, alanine, and proline omitted

*All other ingredients of these media were identical with the formula of medium M 150 (3, 4).

Chromatographic Techniques

Changes in the amino acid content of the synthetic media resulting from cultivation of strain L cells were measured by paper chromatography. The used nutrient fluids from groups of cultures were pooled and 5.0 ml. samples used for analysis. These were concentrated to dryness *in vacuo* over sulphuric acid and reconstituted in 0.2 ml. of deionized water. Five to twenty microliters of this concentrated material were found sufficient for satisfactory analyses, with one-dimensional descending paper chromatograms.

Two solvent systems were employed in these studies: a *n*-butanol – acetic acid – water mixture, and an ethanol–butanol–water mixture, as detailed previously (1), without desalting of the concentrated media. Two general chromogenic reagents for amino acids were used, ninhydrin or isatin. In addition to these two general methods, 12 specific color reactions for individual amino acids were employed. These have been described in detail in a previous publication (2). Full details of the chromatographic procedures, development times, and heating periods have already been reported (1,2). Each of the amino acids present in the synthetic media was dissolved in the basic salt solution and run individually on paper chromatograms, with the two solvent systems employed, in order to establish its relative position on the chromatograms under these experimental conditions.

TABLE II

SUMMARY OF CHANGES IN AMINO ACID COMPOSITION OF VARIOUS SYNTHETIC MEDIA DURING CULTIVATION OF L STRAIN CELLS[a]

Amino acid	Cultivation medium			
	M 150 complete	M 746 glutamic omitted	M 762 glutamine omitted	M 856 glutamic, glutamine omitted
Cystine	**	**	**	*
Lysine	*	*	**	0
Histidine	**	**	**	0
Arginine	*	*	**	0
Glutamine	**	**		
Aspartic acid	0	0	0	0
Serine	+	+	0	0
Hydroxyproline	0	0	0	0
Glycine	+	+	0	0
Glutamic acid	++	++	**	++
Threonine	+	+	*	0
Alanine	0	0	**	0
Proline	+	+	**	0
Tyrosine	+	+	*	+
Tryptophan	*	*	0	0
Methionine	++	++	*	0
Valine	*	*	**	0
Phenylalanine	**	**	**	0
Isoleucine	*	*	**	*
Leucine	**	**	**	*

[a] *=decrease, 0=no change, +=accumulation.

Results

Amino Acid Changes during Cultivation of Strain L Cells in Synthetic Medium M 150

Cultures of strain L cells were prepared as described and carried for an experimental period of 3 weeks in synthetic medium M 150. The twice-weekly fluid changes were pooled, concentrated to dryness, and analyzed on paper chromatograms. The results of several experiments are summarized in Table II, column 1. It is evident that cystine, lysine, histidine, arginine, glutamine, tryptophan, valine, phenylalanine, isoleucine, and leucine have decreased in concentration in the culture medium. Increases were found in the concentrations of serine, glycine, glutamic acid, threonine, proline, and methionine. No changes in the levels of aspartic acid, hydroxyproline, or alanine could be detected. A ninhydrin-positive spot was found on the chromatograms slightly above the tyrosine region. Although a positive identification has not yet been made, the compound appears to be one of the amino butyric acids.

Production of Alanine by Strain L Cells Cultivated in Alanine-Deficient Medium

The observation that strain L cells caused no change in the alanine content of synthetic medium M 150 was of considerable interest, since all freshly explanted tissues so far studied brought about an accumulation of this amino acid in the culture fluids (1, 2). Accordingly, the effect of omitting alanine from the propagation medium was investigated. Since M 894 (Table I)

FIG. 1. Amino acid changes in synthetic medium M 894 (containing no alanine, glutamic acid, or proline) during cultivation of strain L cells. Chromatogram developed with butanol – acetic acid – water, and treated with ninhydrin. LS2 = unused control medium. Columns 1, 2, 3 = used culture media obtained during the successive twice-weekly fluid renewals.

S = Isoleucine
R = Leucine
Q = Phenylalanine
P = Valine
O = Methionine

N = Tryptophan
M = Phenol red
L = Tyrosine
K = Proline
J = Alanine

I = Threonine
H = Glutamic acid

G = Glycine, serine, hydroxyproline
F = Aspartic acid
E = Glutamine

D = Arginine
C = Histidine
B = Lysine

A = Cystine

FIG. 2. Amino acid changes in synthetic medium M 894 (containing no alanine, glutamic acid, or proline) during cultivation of strain L cells. Chromatogram developed with butanol–ethanol–water, and treated with ninhydrin. LS1 = unused control medium. Columns 1 and 2 = used culture media obtained during the successive twice-weekly fluid

M = Isoleucine
Leucine

L = Phenylalanine
Valine

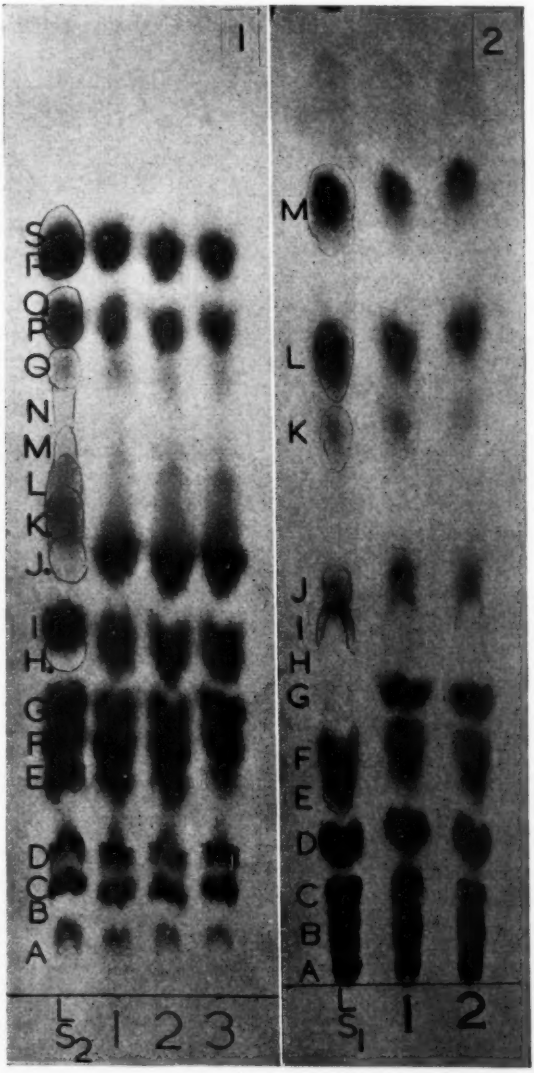
K = Methionine

J = Tryptophan
I = Tyrosine
H = Proline
G = Alanine

F = Threonine
E = Hydroxyproline
D = Glycine, serine

C = Glutamine
B = Basic amino acids
A = Basic amino acids

had produced interesting results in previous studies with chick embryonic heart fibroblasts (9) it was selected as the alanine-deficient medium. The culture fluids from strain L cells cultivated in M 894 were harvested in the usual way and analyzed chromatographically, with the results shown in Figs. 1 and 2.



It is evident (Fig. 1) that cultivation of strain L cells in a medium containing no alanine, glutamic acid, or proline has resulted in two alterations of the chromatographic pattern. A marked accumulation of both alanine (position J) and glutamic acid (position H) has occurred in the used culture fluids (columns 1, 2, and 3). The presence of these amino acids is in marked contrast to their absence in the unused control medium (column LS2). The formation of alanine is illustrated further in Fig. 2, which shows the accumulation of this amino acid in the G region. Proline, which normally would occupy the H region, is completely absent from the control medium and does not appear in the used culture fluids. The neutral solvent system used to develop this chromatogram does not permit separation and resolution of glutamic acid.

Effect of Omission of Glutamic Acid and/or Glutamine on Amino Acid Metabolism of Strain L Cells

Conflicting reports on the necessity of glutamine for propagation of strain L cells (10, 11) made it of interest to determine the effect of omission from the synthetic medium of these two closely related compounds. Accordingly, three synthetic media were prepared from which glutamic acid, glutamine, or both were omitted (Table I). Strain L cultures were prepared in the usual manner and maintained for 3 weeks in each of the three experimental media. Paper chromatographic analyses of the used culture fluids are summarized in Table II.

Strain L cells cultivated in M 746, containing no glutamic acid, show a pattern of amino acid uptake and accumulation indistinguishable from that of cultures in the complete medium M 150 (Table II, column 2). Glutamic acid has accumulated in the culture fluids to approximately the same extent as when the culture medium contained this amino acid initially.

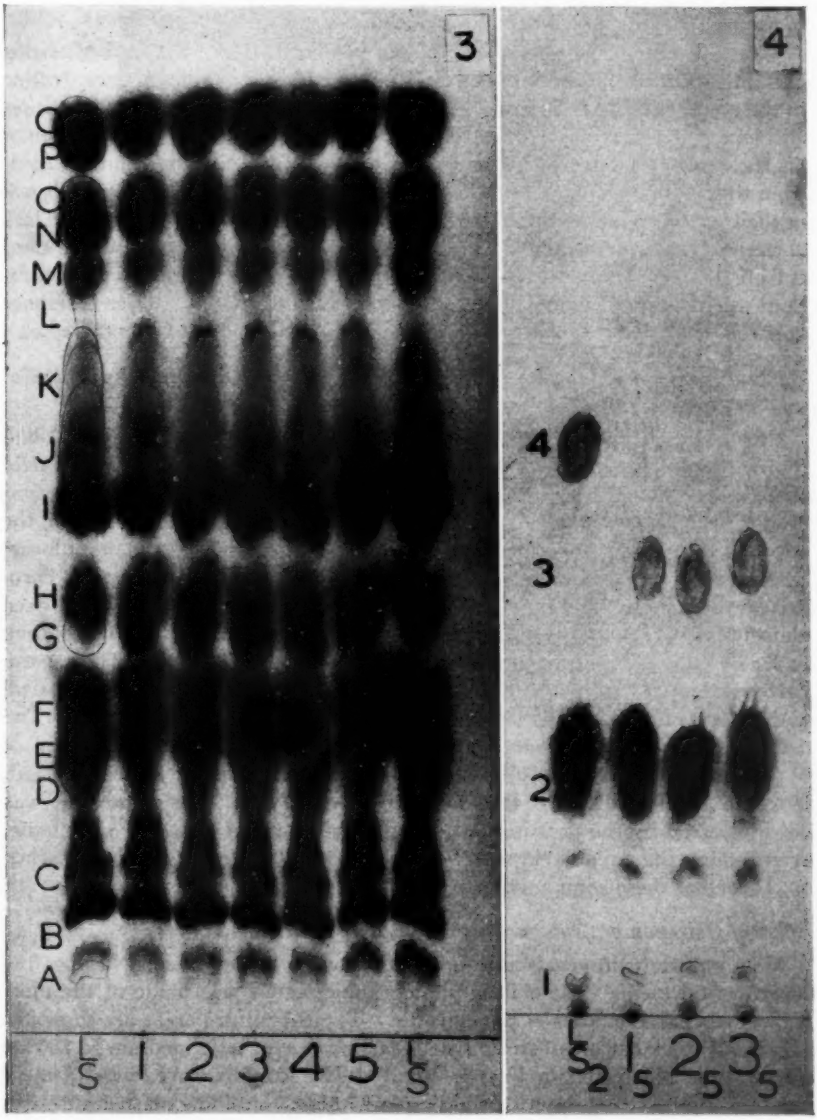
FIG. 3. Amino acid changes in synthetic medium M 856 (containing no glutamine or glutamic acid) during cultivation of strain L cells. Chromatogram developed with butanol – acetic acid – water, and treated with ninhydrin. LS = unused control medium. Columns 1, 2, 3, 4, 5 = used culture media obtained during the successive twice-weekly fluid renewals.

Q = Isoleucine
P = Leucine
O = Phenylalanine
N = Valine
M = Methionine
L = Tryptophan
K = Tyrosine
J = Proline
I = Alanine
H = Threonine
G = Glutamic acid
F = Glycine, serine, hydroxyproline
E = Aspartic acid
D = Glutamine
C = Arginine
B = Lysine
Histidine
A = Cystine

FIG. 4. Changes in adenine content during cultivation of strain L cells in synthetic medium M 150. Chromatogram developed with butanol – acetic acid – water, and treated with chloride reagent. LS2 = unused control medium. Columns 1_5, 2_5, 3_5 = used culture media obtained during the successive twice-weekly fluid renewals.

4 = Adenine
3 = Hypoxanthine
2 = Chloride ions
1 = Inorganic salts

Strain L cells cultivated in M 762, containing no glutamine, showed a completely altered pattern of amino acid changes from that observed in the complete M 150. The decrease of many amino acids in the culture fluids, particularly lysine, arginine, valine, and isoleucine, was noticeably greater. Glutamic acid, which accumulated in all other media studied, disappeared



from this medium, but no formation of glutamine could be demonstrated. Threonine, proline, tyrosine, and methionine, which had accumulated when the cells were cultivated in the complete medium, now were removed from the culture fluids. Serine and glycine no longer increased in the medium, but appeared to be unchanged in relative concentration. In general, the removal of amino acids from the medium appeared to be stimulated and accumulation of amino acids in the culture fluids prevented.

When strain L cells were cultivated in medium M 856, containing neither glutamic acid nor glutamine, almost no changes in amino acid concentration in the medium could be detected. Only three amino acids, cystine, isoleucine, and leucine, were removed from the culture fluids, and these to only a minor degree, while a moderate accumulation of glutamic acid was found. Aside from these minor changes, both decrease and accumulation of amino acids was negligible. A typical chromatogram, illustrating the almost complete lack of activity of the cultures, is shown in Fig. 3. It is evident that the used culture fluids (columns 1 to 5) show essentially the same amino acid concentrations as the unused control medium (column LS). The only major difference apparent is the formation of glutamic acid in the experimental fluids (G region).

Effect of Omission of Glutamic Acid and/or Glutamine on Metabolism of Adenine by Strain L Cells

Previous experiments with a variety of freshly explanted tissues (1, 2) had shown that each type of culture exhibited a different pattern of amino acid changes in the medium, but that a consistent decrease in the adenine content of the medium occurred. Accordingly, the effect of strain L cells on the adenine content of the five test media (Table I) was studied, using the chloride reagent of Wood (12) to detect adenine and hypoxanthine. A typical chromatogram is shown in Fig. 4. It is evident that adenine (position 4) has been almost completely removed from the medium and that the spot corresponding to the location of hypoxanthine (position 3) has been intensified. The general pattern of changes brought about by strain L cells appeared to be identical with that of the freshly explanted tissues examined previously (1, 2). The decrease in adenine content of the medium and the probable formation of hypoxanthine by strain L cells was found to occur in all five media tested. It would appear, therefore, that the activity of strain L cells on adenine was not influenced by the presence or absence of glutamic acid and/or glutamine, even though the amino acid metabolism was greatly affected by the presence or absence of these compounds.

Effect of Omission of Glutamine on Survival of Strain L Cultures

At appropriate intervals during cultivation of strain L cells in the five experimental media (Table I), a photographic record was made of the morphological characteristics of the cultures. Typical culture areas are shown in Fig. 5. A stock culture of strain L cells carried in synthetic medium M 150 for 5 months is illustrated in Fig. 5, No. 1. The same culture, subsequently photographed after 34 days' cultivation in M 856 (containing neither glutamic

PLATE I

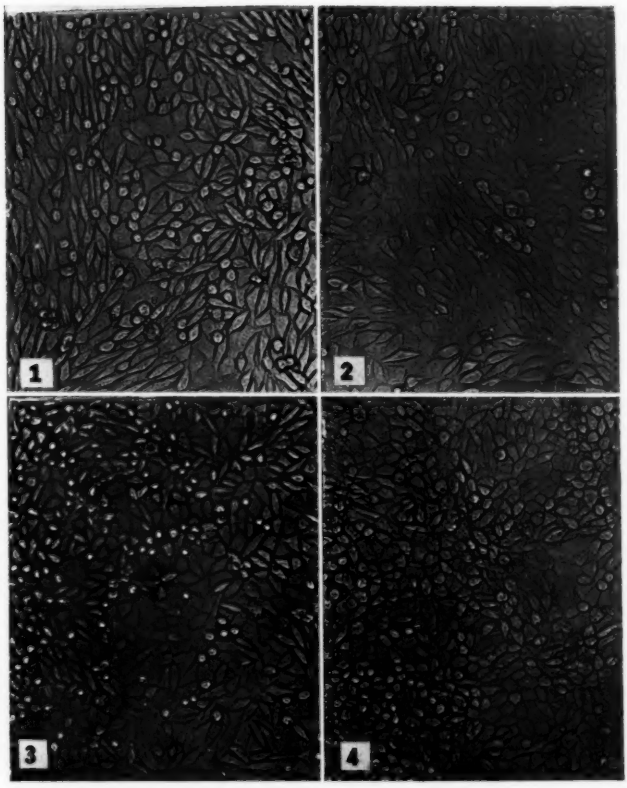


FIG. 5.

No. 1. Strain L cells after 5 months' continuous cultivation in synthetic medium M 150. ×105
No. 2. Same culture area as Fig. 1 subsequently photographed after 34 days' cultivation in M 856 (containing neither glutamic acid nor glutamine). ×105
No. 3. Strain L cells after 20 days' cultivation in M 416. ×105
No. 4. Same culture area as Fig. 3 subsequently photographed after 10 days' cultivation in M 762 (containing no glutamine). ×105

acid nor glutamine) is shown in Fig. 5, No. 2. A 20-day old culture in synthetic medium M 416 is shown in Fig. 5, No. 3. The same culture, subsequently photographed after 10 days in M 762 (containing no glutamine) is shown in Fig. 5, No. 4. It appears that neither short-term cultivation in a glutamine-deficient medium nor long-term cultivation in a medium lacking both glutamine and glutamic acid caused a reduction in cell population or degenerative changes in the cells. Similar results were obtained with repeated experiments in the five test media over the 3-week experimental period. It should be noted in this connection that previous studies (13) have established that a depletion period of 3 days is sufficient to reduce the stores of intracellular nutrients and intensify the tissue culture response to an essential metabolite. The results illustrated in Fig. 5 suggest that glutamine is not essential for survival of strain L cells under the present experimental conditions, even though this amide had been reported previously (10, 14) as essential for the propagation of strain L cells in media supplemented with dialyzed serum. The possibility remains, however, that glutamine may be required for rapid propagation in addition to survival under the present conditions.

Discussion

Since the original demonstration by Sanford, Earle, and Likely (15) that cell lines may be derived through single-cell isolation, strain L Clone 929 cells have found wide application in tissue culture research. Early media used for the cultivation of these cells contained whole or dialyzed horse serum (14, 16), but it has recently been shown that long-term cultivation is possible in completely synthetic media (17). In the present experiments, it was found that strain L cells could be cultivated for at least 6 months in completely synthetic medium M 150 (3, 4) or M 416 (5) without serious degeneration (Fig. 5, No. 1). To maintain a rapid rate of cell propagation in the stock cultures, however, brief supplementation with horse serum was found necessary at regular intervals. While propagation of the cells was not maximal under these conditions, both survival rate and metabolic activity were found to be high. During the experimental periods, the cell population in each flask increased moderately, as evidenced by extension of the monolayer to completely cover the glass surface, and no cell degeneration was detected (Fig. 5). The resulting patterns of amino acid changes in the culture medium are, therefore, attributed to cell metabolism under conditions of survival and maintenance.

Rapid growth of cell strains in semidefined media (10, 14) has been employed to determine the specific nutritional requirements of cells. It has recently been shown, however, that such cell lines in continuous cultivation become altered in morphology (18) and malignant in character (19, 20), even though derived originally from normal tissues. Accordingly, the use of these techniques does not permit comparisons to be made of the nutrition and metabolism of normal and malignant tissues under comparable conditions. For this reason, the present studies with the malignant strain L cell were deliberately carried out under conditions of survival and maintenance, as distinct from

rapid propagation. The use of these conditions has made it possible to compare the metabolic activities of strain L cells with the amino acid patterns previously established for fresh explants of a variety of normal tissues (1, 2).

In general, the amino acid metabolism of strain L cells appeared to differ from that of freshly explanted tissues (1, 2) in showing less decrease in the basic amino acid region and greater decrease in the phenylalanine and isoleucine regions. In addition, strain L cells showed a moderate decrease of glutamine in the culture medium while freshly explanted chick embryonic tissues tended to accumulate this amide in their environment. This observation is of interest in view of the demonstration by Roberts and Borges (21) that glutamine is released into the surrounding fluids by regressing but not by actively growing tumors. Recent studies with HeLa cells (to be published) have also shown decrease of glutamine from the culture medium without apparent formation of glutamic acid. These observations raise the possibility that a difference between normal and malignant cells in tissue culture may exist in respect to the metabolism of glutamine, and further studies to investigate this possibility are in progress.

Glutamic acid and alanine are not considered as essential amino acids for strain L cells (14). In the present experiments, glutamic acid was observed to accumulate in the culture medium, while the level of alanine was unchanged. However, omission of alanine from the synthetic medium resulted in its accumulation in appreciable amounts. Similarly, omission of glutamic acid from the medium resulted in its formation in considerable quantity. These observations are of interest in view of the recent demonstration by Sanford and co-workers (11) that the nonessential amino acids are necessary to maintain maximum proliferation of strain L cells.

In the present experiments, omission of glutamic acid from the synthetic medium, which still contained glutamine, caused no appreciable alteration of the pattern of amino acid decrease and accumulation, but the omission of glutamine, in the presence of glutamic acid, greatly altered the amino acid metabolism pattern. Serine and glycine, which had previously increased slightly in concentration, now showed no change. Glutamic acid, threonine, proline, tyrosine, and methionine, which previously had accumulated, were now removed from the culture medium. Alanine, which had shown no change in concentration previously, was now removed from the medium to an appreciable extent. In general, omission of glutamine appeared to stimulate removal of nearly all amino acids from the culture medium and to block the metabolic pathways leading to accumulation of amino acids in the medium. Omission of *both* glutamic acid and glutamine resulted in an even more drastic alteration of the metabolic pattern. With a few minor exceptions, both amino acid decrease and accumulation in the culture medium virtually ceased.

The present results suggest strongly that glutamine and glutamic acid play a major role in regulating the amino acid metabolism of strain L cells. Such a concept is not entirely new. Earlier experiments by Westfall and co-workers (22) had shown that in the absence of glutamine two strains of liver cells were

unable to utilize glucose or amino acids from the culture medium and died rapidly. Ascitic tumor cells have been shown to accumulate amino acids from the surrounding fluids (23) and the incorporation of all amino acids was found to be stimulated by glutamine (24). In animal experiments, the feeding of glutamic acid (25), alone of the amino acids studied, resulted in a decreased level of plasma amino acids and an increase in cellular amino acids. The present observations are unusual, however, in indicating entirely different effects on amino acid metabolism when glutamic acid, glutamine, or both are omitted from the culture medium. It is evident that, in studies of tissue culture nutrition and metabolism, the nonessential, as well as the essential, amino acids must receive careful consideration.

In contrast to the demonstrated effect of glutamic acid and/or glutamine in regulating amino acid metabolism of strain L cells, the presence *or* absence of these compounds appeared to have no effect on the cell activity towards adenine. Decrease in adenine and production of hypoxanthine was found to be quite independent of the amino acid content of the culture medium. It is evident, moreover, that although the amino acid metabolism patterns are different with each type of tissue culture examined (1, 2) the activity of both freshly explanted tissues and cell strains towards adenine appears to be similar. These findings are in accord with the observations of Lu and Winnick (26) on the uptake of C^{14}-labelled adenine by tissue cultures and the suggestion that this uptake be used as a criterion of cell proliferation (27).

Present data on the role of glutamine in tissue culture nutrition are somewhat conflicting. Early experiments, in media containing serum protein, suggested that glutamine was essential for growth of strain L (14) and HeLa cells (28). It was found (10) that high levels of glutamic acid would replace the glutamine requirement of the HeLa cell but not of strain L cells. Glutamine has also been shown to be a precursor of asparagine for the HeLa cell (29) and to play a role in protein synthesis by this cell line (30). Experiments with freshly explanted chick embryonic heart cultures in completely synthetic media (9) established that glutamine was not required for survival of these cultures. Recent experiments with strain L cells in completely synthetic media (11) have shown that glutamine is not an essential factor for growth but that this amide, together with the nonessential amino acids, helps to maintain propagation at a maximal rate. The results of the present experiments are in general agreement with the findings of Sanford and co-workers (11) that glutamine is not an essential factor for survival of strain L cells although it may be required for rapid propagation. The demonstrated effect of this compound on over-all amino acid metabolism, however, suggests strongly that it should be incorporated in chemically defined growth media for strain L cells.

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References

1. PASIEKA, A. E., MORTON, H. J., and MORGAN, J. F. J. Natl. Cancer Inst. **16**, 995 (1956).
2. PASIEKA, A. E., MORTON, H. J., and MORGAN, J. F. Can. J. Biochem. and Physiol. **36**, 171 (1958).
3. MORGAN, J. F., CAMPBELL, M. E., and MORTON, H. J. J. Natl. Cancer Inst. **16**, 557 (1955).
4. MORGAN, J. F., MORTON, H. J., and PARKER, R. C. Proc. Soc. Exptl. Biol. Med. **73**, 1 (1950).
5. MORGAN, J. F., MORTON H. J., CAMPBELL, M. E., and GUERIN, L. F. J. Natl. Cancer Inst. **16**, 1405 (1956).
6. EARLE, W. R. and HIGHHOUSE, F. J. Natl. Cancer Inst. **14**, 841 (1954).
7. MORGAN, J. F. and MCCRONE, M. A. J. Natl. Cancer Inst. **19**, 393 (1957).
8. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J. J. Biol. Chem. **193**, 265 (1951).
9. MORGAN, J. F. and MORTON, H. J. J. Biophys. Biochem. Cytol. **3**, 141 (1957).
10. EAGLE, H., OYAMA, V. I., LEVY, M., HORTON, C. L., and FLEISCHMAN, R. J. Biol. Chem. **218**, 607 (1956).
11. SANFORD, K. K., MCQUILKIN, W. T., EVANS, V. J., and EARLE, W. R. J. Natl. Cancer Inst. In press.
12. WOOD, T. Nature, **176**, 175 (1955).
13. MORTON, H. J., PASIEKA, A. E., and MORGAN, J. F. J. Biophys. Biochem. Cytol. **2**, 589 (1956).
14. EAGLE, H. J. Biol. Chem. **214**, 839 (1955).
15. SANFORD, K. K., EARLE, W. R., and LIKELY, G. D. J. Natl. Cancer Inst. **9**, 229 (1948).
16. SANFORD, K. K., WESTFALL, B. B., FIORAMONTI, M. C., MCQUILKIN, W. T., BRYANT, J. C., PEPPERS, E. V., EVANS, V. J., and EARLE, W. R. J. Natl. Cancer Inst. **16**, 789 (1955).
17. EVANS, V. J., BRYANT, J. C., FIORAMONTI, M. C., MCQUILKIN, W. T., SANFORD, K. K., and EARLE, W. R. Cancer Research, **16**, 77 (1956).
18. PARKER, R. C., CASTOR, L. N., and MCCULLOCH, E. A. Anat. Record, **124**, 18 (1956).
19. BENDER, M. A. Science, **126**, 974 (1957).
20. HSU, T. C., POMERAT, C. M., and MOORHEAD, P. S. J. Natl. Cancer Inst. **19**, 867 (1957).
21. ROBERTS, E. and BORGES, P. R. F. Cancer Research, **15**, 697 (1955).
22. WESTFALL, B. B., EVANS, V. J., PEPPERS, E. V., HAWKINS, N. M., and EARLE, W. R. J. Natl. Cancer Inst. **17**, 131 (1956).
23. CHRISTENSEN, H. N., RIGGS, T. R., FISCHER, H., and PALATINE, I. M. J. Biol. Chem. **198**, 1 (1952).
24. RABINOVITZ, M., OLSON, M. E., and GREENBERG, D. M. J. Biol. Chem. **222**, 879 (1956).
25. CHRISTENSEN, H. N., STREICHER, J. A., and ELBINGER, R. L. J. Biol. Chem. **174**, 515 (1948).
26. LU, K. H. and WINNICK, T. Exptl. Cell Research, **6**, 345 (1954).
27. LU, K. H. and WINNICK, T. Exptl. Cell Research, **7**, 238 (1954).
28. EAGLE, H. J. Exptl. Med. **102**, 37 (1955).
29. LEVINTOW, L. Science, **126**, 611 (1957).
30. LEVINTOW, L., EAGLE, H., and PIEZ, K. A. J. Biol. Chem. **227**, 929 (1957).

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(ii) ***Line drawings***

Drawings should be carefully made with India ink on white drawing paper, blue tracing linen, or co-ordinate paper ruled in blue only; any co-ordinate lines that are to appear in the reproduction should be ruled in black ink. Paper ruled in green, yellow, or red should not be used. All lines should be of sufficient thickness to reproduce well. Decimal points, periods, and stippled dots should be solid black circles large enough to be reduced if necessary. Letters and numerals should be neatly made, preferably with a stencil (**do NOT use typewriting**), and be of such size that the smallest lettering will be not less than 1 mm. high when reproduced in a cut 3 in. wide.

Many drawings are made too large; originals should not be more than 2 or 3 times the size of the desired reproduction. Wherever possible two or more drawings should be grouped to reduce the number of cuts required. In such groups of drawings, or in large drawings, full use of the space available should be made; the ratio of height to width should conform to that of a journal page (4½ × 7½ in.), but allowance must be made for the captions.

The original drawings and one set of clear copies (e.g. small photographs) are to be submitted.

(iii) ***Photographs***

Prints should be made on glossy paper, with strong contrasts. They should be trimmed so that essential features only are shown and mounted carefully, with rubber cement, on white cardboard, with no space between them. In mounting, full use of the space available should be made to reduce the number of cuts required (see Illustrations (ii)). Photographs or groups of photographs should not be more than 2 or 3 times the size of the desired reproduction.

Photographs are to be submitted in duplicate; if they are to be reproduced in groups one set should be mounted, the duplicate set unmounted.

Reprints

A total of 50 reprints of each paper, without covers, are supplied free. Additional reprints, with or without covers, may be purchased at the time of publication.

Charges for reprints are based on the number of printed pages, which may be calculated approximately by multiplying by 0.6 the number of manuscript pages (double-spaced typewritten sheets, 8½ × 11 in.) and including the space occupied by illustrations. Prices and instructions for ordering reprints are sent out with the galley proof.

Any reprints required in addition to those requested on the author's reprint requisition form must be ordered officially as soon as the paper has been accepted for publication.

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